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CANINE CHRONIC INFLAMMATORY ENTEROPATHIES: DIAGNOSTIC MARKERS, ENDOSCOPIC TECNIQUES AND NOVEL THERAPEUTIC APPROACHES

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ABSTRACT

Canine chronic inflammatory enteropathies (CIE) are a group of diseases characterized by chronic gastrointestinal (GI) symptoms. Once other causes for GI symptoms have been ruled out, subdivision of CIEs occurs through therapeutic response which involves a unique trial. The therapeutic steps include a dietary change, subsequently the introduction of antibiotic therapy (this step has recently been discouraged), the execution of an endoscopy of the digestive tract and the introduction of an immunosuppressive therapy. Understanding the pathogenesis and the type of CIE in is fundamental for a correct diagnostic and therapeutic management. There are also several laboratory prognostic markers and clinical scores to help the clinician understand the type of CIE and the severity of the patient's clinical condition. Research in the field of dog CIE is in continuous development with the production of new therapeutic and management indications.

The aim of this research was to rewire current literature about CIE, with particular focus on diagnostic and therapeutic aspects. Experimental studies, conducted during author's PhD, were reported. Studies were focused on diagnostic markers (page 13), GI endoscopy (page 57, page 67) and therapeutic management (page 88) of canine CIE.

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NOMENCLATURE

CIE	Chronic Inflammatory Entropathy
FRE	Food Responsive Enteropathy
ARE	Antibiotic Responsive Enteropathy
IRE	Immunosuppressant Responsive Enteropathy
PLE	Protein-Losing Enteropathy
NRE	Non-Responsive Enteropathy
AA	Amino Acids
ACTH	Adenocorticotropic Hormone
AFR	Adverse Food Reaction
ALT	Alanine Aminotransferase
ARD	Antibiotic Responsive Diarrhea
AST	Aspartate Aminotransferase
AT III	Antithrombin III
BA	Bile Acids
BARF	Bone and Raw Food Diet
BCS	Body Condition Score
BVS	Bilious Vomiting Syndrome
BW	Body Weight
CCECAI	Canine Chronic Enteropathy Clinical Activity Index
CD	Chron's Disease
CIBDAI	Canine IBD Activity Index
cPLI	Canine Pancreatic Lipase Immunoreactivity
CRP	C-Reactive Protein
EAS	Endoscopic Activity Score
EPI	Exocrine Pancreatic Insufficiency
FA	Food Allergy
FISH	Flourescent in Situ Hybridization
FMT	Fecal Microbiota Transplantation
FOS	Fructooligosaccharides
FS	Fecal Score
GALT	Gut Associated Lymphoid Tissue
GD	Gastro-duodenal endoscopy
GDIC	Gastro-duodenal-ileum-colonoscopy
GI	Gastrointstinal
GSD	German Shepherd Dog
HUC	Histiocytic Ulcerative Colitis
HYC	Homocysteine
IBD	Inflammatory Bowel Disease
Ig	Immunoglobuline
IHC	Immunohistochemistry
IL	Interleukine
MOS	Mannooligosaccharides
Nf-kB	Nuclear factor-kappa B
NLR	Neutrophil-to-Lymphocyte Ratio
NOD	Nucleotide-Oligomerization Domain
PARR	Polymerase Chain Reaction
PLN	Protein Losign Nephropathy
PO	Oral Route
PRRs	Pattern Recognition Receptors
PU/PD	Polyuria Polydipsia

rRNA	Ribosomial Ribonucleic Acid	TLR	Toll Like Receptor
SAP	Serum Alkaline Phosphatase	TNF	Tumor Necrosis Factor
SC	Subcutaneous	UC	Ulcerative Colitis
SCWT	Soft Coated Weaton Terrier	UPC	Urinary Protein to Creatinine Ratio
Th	Lymphocite Helper T	α1-PI	α1 Protease Inhibitor
TLI	Trypsine-Like Immunoreactivity	(q)PCR	(quantitative) Polymerase Chain Reaction

INTRODUCTION

Chronic Inflammatory Enteropathies (CIE) are a group of gastrointestinal (GI) disease that can affect dogs characterized by chronic symptoms, persistent or recurrent for at least three weeks, such as vomiting, diarrhea, weight loss, anorexia, pica [Procoli, 2020]. The term Inflammatory Bowel Disease (IBD) is often used to refer to CIE. IBD is a chronic human disease characterized by gastrointestinal symptoms that include two forms: Chron's disease (CD) and Ulcerative colitis (UC). The analogy is due to the similar pathogenesis between canine CIE and human IBD, in particular host genetics, aberrant immune response and chronic inflammation. However, the clinical course, therapeutic management and prognosis are different between these diseases, so it would be better not to use the same acronym (IBD) to identify the disease in humans and dogs [Simpson & Jergens, 2011; Dandrieux, 2016].

Once non-gastrointestinal causes for these symptoms are excluded, the classification of canine CIE is based on clinical response to the therapeutic trial and are divided into: Food-Responsive Enteropathy (FRE), Antibiotic Responsive Enteropathy (ARE), Immunosuppressant-Responsive Enteropathy (IRE), Non-Responsive Enteropathy (NRE). A further category, based not on therapeutic trial but on serum albumin value at the time of diagnosis, is Protein-Losing Enteropathy (PLE) [Dandrieux, 2016].

To distinguish between the different forms of CIE, various diagnostic investigations are envisaged. In subjects unresponsive to the first two steps, diet and antibiotic, an endoscopy of the digestive tract is performed to evaluate the gastrointestinal tract and obtain biopsies [Washabau et al., 2010]. According to the most recent indications, the step with antibiotic is not recommended with greater use of probiotics in place of antibiotics and development of new therapeutic approaches, such as fecal transplantation [Cerquetella et al., 2020; Chaitman & Gaschen, 2020; Procoli, 2020].

1 PATHOGENESIS

Pathogenesis of CIE is multifactorial and due to an interaction between host genetics, intestinal microbiota, immune system, dietary constituents, and environmental factors. The interaction between these factors leads to chronic intestinal inflammation associated with the typical clinical symptoms of CIE [Allenspach et al., 2010; Simpson & Jergens, 2011; Dandrieux, 2016].

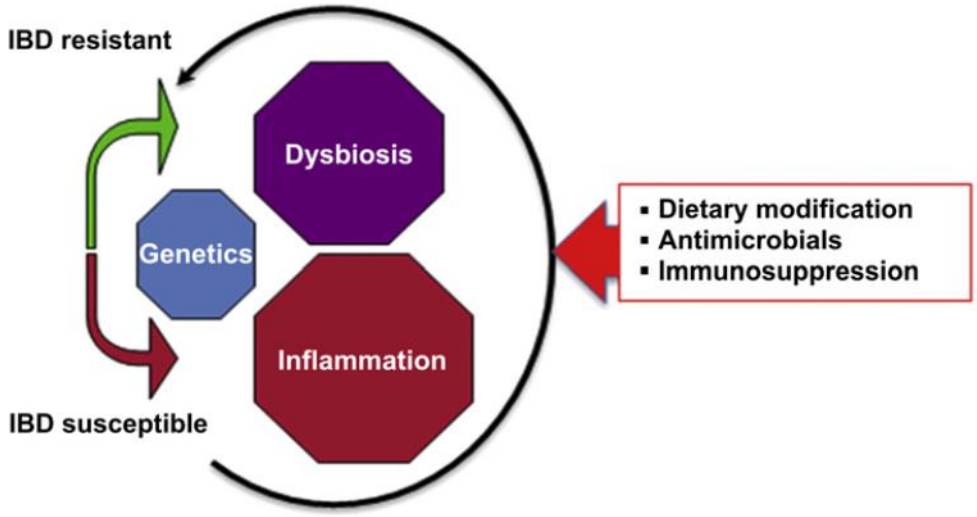

Figure 1.a: Factors involved in the pathogenesis of canine IBD. Modified from: Simpson & Jergens, 2011.

1.1 IMMUNE SISTEM AND GENETIC

The mucosal surface of the GI tract is continuously exposed to antigenic stimuli. In a healthy GI tract, there is a balance between the release of pro- and anti-inflammatory mediators, and if this balance is broken an aberrant inflammatory response can occur [Atreya et al., 2008]. To regulate the response correctly toward food antigens and host microbiota (oral tolerance) rather than towards pathogens, two mechanisms are involved: innate immunity and adaptive immunity [Gaschen & Merchan, 2011; Geremia et al., 2014].

Innate immunity is given by the physical barrier, formed by enterocytes with their tight junctions, peristaltic movements, mucus, microflora, macrophages, dendritic cells and some subtypes of lymphocytes T. The integrity of the intestinal barrier and the correct interaction between these mechanisms guarantees protection against pathogens (Figure 1.c) [Eissa et al., 2019]. The recognition of molecules, commensals or pathogenic, occurs through receptor, the Pattern Recognition Receptors (PRRs). A particular type of PRRs are the Toll-Like Receptors (TLRs), which play a fundamental role in the GI apparatus as they recognize some molecules expressed by bacteria and consequently stimulate further factors [Kawai & Akira, 2010]. Exposure

to antigenic stimuli involves, through intracellular signals, the production of Nuclear factor-kappa B (Nf-kB) which promotes the release of pro-inflammatory cytokines and interleukins such as Tumor Necrosis Factor alpha (TNF-alpha), IL-1, IL-6, IL-8 [Atreya et al., 2008]. Other PRRs involved in the pro-inflammatory response are Nucleotide-Oligomerization Domain (NOD) [Aono et al., 2019]. In healthy individuals, there is a correct activation of these receptors with consequent expression of pro- and anti-inflammatory mediators [Abreu, 2010]. In dogs with CIE, TLRs, Nf-kB and NODs were found to be altered in expression, and therefore there is an over-expression of pro-inflammatory mediators. These receptors are therefore strongly involved in the pathogenesis of CIE, but exact mechanism remain unclear. A genetic predisposition was demonstrated in German Shepherd dog (GSD) and some other breeds for genes encoding some TRLs, confirming the importance of genetics in the immune-mediate mechanisms of CIE [Atreya et al., 2008; Burgener et al., 2008; Allenspach et al., 2010; Kathrani et al., 2010; Aono et al., 2019].

Adaptive immunity is a specific and long-lasting immunity that is the result of maturation and development of immune cells after antigenic stimuli given by a lymphoid tissue, the Gut Associated Lymphoid Tissue (GALT) [Geremia et al., 2014; Eissa et al., 2019]. The antigenic stimulus involves the differentiation of B and T lymphocytes whose main actions are respectively the production of Immunoglobulins (Ig), in particular IgA, and the elimination of pathogens and infected cells [Geremia et al., 2014]. IgA are found on the mucosal surface and prevent pathogenic bacteria, viruses and parasites from penetrating the intestinal barrier. IgA's production is strictly related to the intestinal microbiota and their role is to avoid an aberrant reaction toward normal component of the microflora [Suzuki et al., 2007]. For these mechanisms, a deficiency of IgA can favor an alteration of intestinal homeostasis. The concentration of IgA can vary for several causes, such as age, but it is known that in dogs with CIE the concentration of intestinal IgA is lower than healthy dogs [Zaine et al., 2011; Maeda et al., 2013; Lee et al., 2015; Grellet et al., 2016]. The main role in the intestinal immune response is played by T lymphocyte that develop and differentiate according to the antigenic stimuli received [Geremia et al., 2014]. Among all types of T cells, those mainly involved in the pathogenesis of human IBD are lymphocytes Helper T 1 (Th1), Helper T 2 (Th2), Helper T 17 (Th17) and Regulatory T cell (Tregs) [Boden & Lord, 2017]. Th1, Th2, Th17 have a pro-inflammatory role against different pathogens, so their abnormal activation can prolong the inflammation with several effect on both adaptive and innate immune system [Geremia et al., 2014]. In dogs, a correlation between CIE and distinct Th lymphocyte profile has not been demonstrated [Heilmann & Suchodolski, 2015]. Tregs have an anti-inflammatory role with the production of cytokines and molecules that inhibit a prolonged and excessive immune response and therefore participate in what is called oral tolerance [Junginger et al., 2012]. Dogs with CIE have a reduced number of Tregs, thus suggesting that a lack of these cells may be related to chronic inflammatory disorders [Junginger et al., 2012; Maeda et al., 2016].

1.2 INTESTINAL MICROBIOTA

The intestinal microbiota is a complex population of microorganisms whose role is relevant in health and disease conditions at the level of the gastrointestinal system. It includes protozoa, viruses, archaea, bacteria, fungi, although in veterinary medicine with the term “microbiota” frequently refers strictly to the bacterial population as it is the most studied and known [Redfern et al., 2017]. The bacterial concentration and diversity, among the GI tract, gradually increases proceeding from the oral cavity to the ileo-colic valve reaching the maximum number in the colon (Figure 1.b) [Hooda et al., 2012]. Although the presence of bacterial groups is constant in animals, there is a great variability of genus and species among different individuals, in fact every single animal has its own and unique microbial ecosystem [Grześkowiak et al., 2015]. The microbial genome confers metabolic capacities superior of those of the host organism, making the intestinal microbiome (genetic heritage that characterize the microbiota) an active participant in the physiology of the host [Barko et al., 2018]. Some processes in which the microbiota intervenes are the production of some vitamins, the turnover of erythrocytes and interaction with the immune system (GALT), the production of short-chain fatty acids, the production of anti-inflammatory cytokines, confirming the importance of a balanced microbial ecosystem for the regulation of host health and immunity (Figure 1.c) [Redfern et al., 2017].

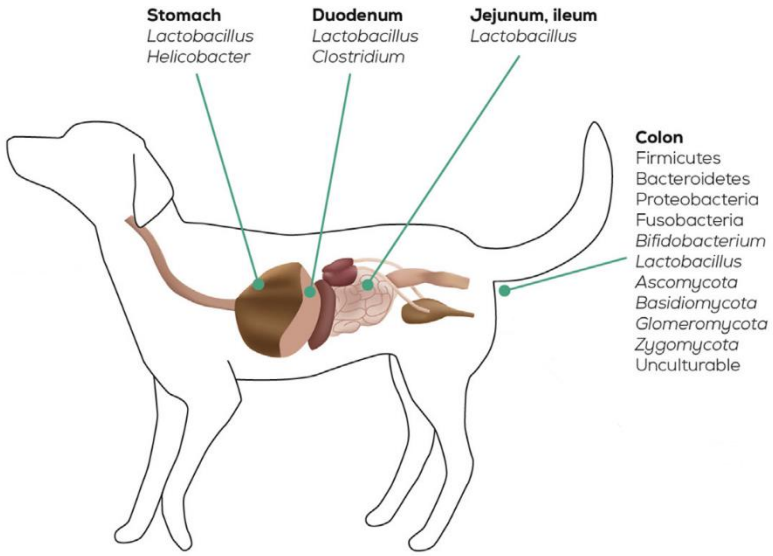

Figure 1.b: The canine gastrointestinal tract and its predominant microorganisms. Modified from: Grzeskowiak et al., 2015.

Various endogenous and exogenous factors, for examples age, diet, antibiotic therapy, environmental factors, can affect the microbiota, creating an alteration of the balance between the microbial populations present with a consequent imbalance that takes the name of intestinal dysbiosis [Honneffer et al., 2014]. Intestinal dysbiosis has been found in dogs with acute or chronic GI disease, however it is not clear whether dysbiosis is a cause or a consequence of these diseases [Tizard & Jones, 2018; Ziese et al., 2018]. Inflammation resulting from GI alteration, in fact, can cause intestinal dysbiosis but, in genetically susceptible individuals, the dysbiosis itself can exacerbate inflammation (Figure 1.c) [Suchodolski, 2016; Eissa et al., 2019]. Alterations on the bacterial component of microbiome have an impact on the production of metabolites in the intestinal lumen with implications also on clinical symptoms representing a stimulus to continuous researches on its composition, activities and therapeutic management in dogs with GI alterations [Guard et al., 2015; AlShawaqfeh et al., 2017; Ziese et al., 2018; Minamoto et al., 2019; Niina et al., 2019; Pilla & Suchodolski, 2020; Werner et al, 2020].

The characterization of intestinal microbiota, to detect alterations in the bacterial composition, is carried out on intestinal samples or fecal samples [Allenspach et al., 2010; Honneffer et al., 2014; Minamoto et al., 2015]. A bacterial culture can provide useful information only in certain cases (e.g. bacterial infections, antibiotic resistance), but in most cases it does not provide relevant information because not all bacteria survive outdoors and are cultured [Suchodolski, 2016; Werner et al., 2020]. Given the complexity of gut microbiota, there is no gold standard technique for assessing it. Among the methods used there are PCR amplification of 16s rRNA genes using broad universal bacteria primers, direct quantification of specific bacterial taxa by quantitative PCR (qPCR), fluorescent in situ hybridization (FISH) to visualize bacterial translocation into mucosal epithelium [Suchodolski, 2016].

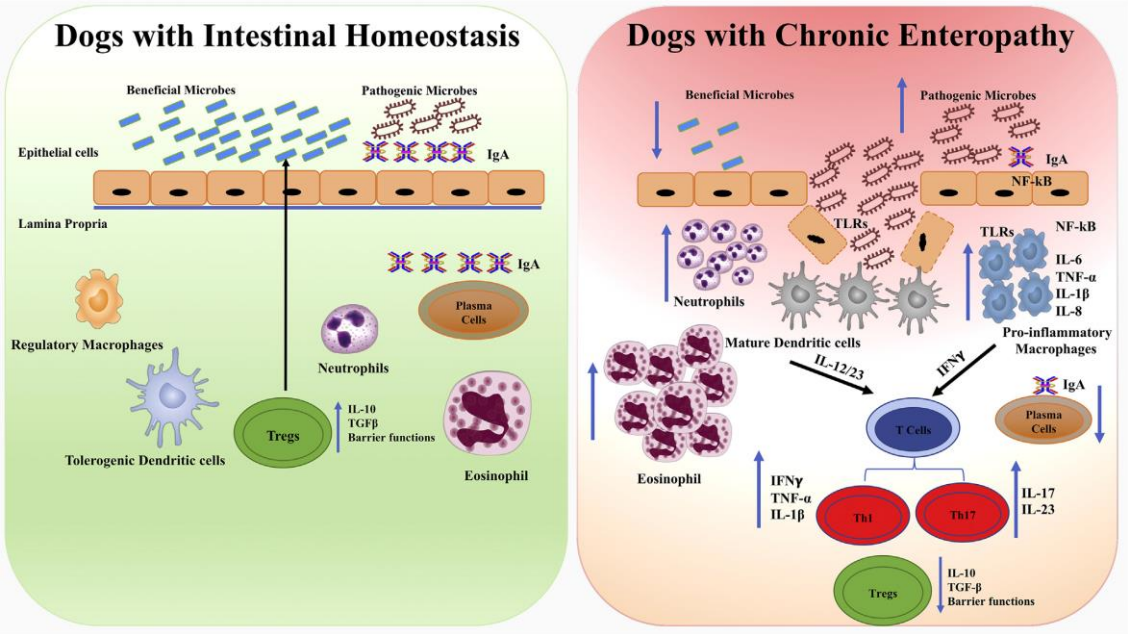

Figure 1.c: Interaction between the mucosal immune system and the intestinal microbiota. On the left side, in healthy dogs, the intestinal microbiota intervenes on the mucosal immune response by promoting immune maturation, strengthening the integrity of the barrier and providing resistance against pathogens. Additionally, it can perform anti-inflammatory activities, through the production of IgA, together with the expansion of Tregs, tolerogenic dendritic cells and regulatory macrophages, which are of great importance for the prevention of immunopathology. On the right side of this figure, there is a hypothetical scenario of dogs with CIE. In this case microbial dysbiosis occurs as a result of the loss of protective bacteria and/or the proliferation of pathogenic microbes with recruitment and activation of inflammatory response characterized by mucosal infiltration of neutrophils, eosinophils, mature dendritic cells, pro-inflammatory macrophages, Th1 and Th17 cells. A strong inflammatory response has its benefits in term of immune and tissue healing but, if uncontrolled, could lead to immunopathology in a genetically sensitive host. Modified from: Eissa et al., 2019.

1.3 DIET AND ENVIROMENTAL FACTORS

Numerous causes are included among the environmental factors involved in the pathogenesis of CIEs, including the dietary components, previous infections or parasites, lifestyle and stress.

The importance of dietary role in CIE is well known, to the point that the term Food-Responsive Enteropathy (FRE) was coined [Dandrieux, 2016]. Adverse Food Reactions (AFRs) are clinically abnormal responses to a dietary component which can manifest itself with GI and/or cutaneous symptoms. They can be immunological reactions, and in this case we define Food Allergy (FA), or non-immunological, and in this case is called Food Intolerance, or, moreover, can be toxic reactions, in this case called intoxications. An adequate

oral tolerance is normally required to prevent AFRs, and consequently an intact mucosal barrier, a balanced microbiota and an adequate GALT response (Figure 1.c). The alteration of one of these systems can therefore favor the establishment of an adverse reaction toward inert foods [Gaschen & Merchant, 2011; Craig, 2019]. There is also a close relationship between ingested food and microflora composition, in fact different foods can influence composition, number and metabolic activity and products of bacteria [Bresciani et al., 2018; Mori et al., 2019; Pilla & Suchodolski, 2020; Rossi et al., 2020b]. While for FA the mechanism is an immunological reaction, and eliminating the antigenic stimulus resolves the aberrant immune response, the pathogenetic mechanisms of food intolerances and the role that they play in CIEs is not completely clear [Craig, 2019].

Among the predisposing factors for CIEs, some infectious and parasitic disease, in particular parvovirus infections and giardiasis, should be mentioned. The suspected mechanism is that these infectious agents with their actions cause damage to the mucosal barrier with consequent loss of oral tolerance, but further studies are needed [Tangtrongsup & Scorza, 2010; Kilian et al., 2018; Perrucci et al., 2020].

Not entirely clear is the role that stress and physical activity can have in the development of CIEs. As in human beings, it seems to be a relation between stressful conditions and GI symptoms in dogs, but the exact mechanism is still unknown [Stavisky et al., 2011; Rose et al., 2017; Cerquetella et al., 2018]. A predisposition to gastrointestinal alterations associated to intense physical activity, in particular gastritis, gastric erosion, bleedings, diarrhea, that is reported in human athletes [Paluska, 2009] and in racehorses [Murray et al., 1996], has also been found in dogs, but even in this case the precise mechanism remains unknown. No infectious or parasitic causes related to the development of these symptoms were found, suggesting that they are linked to an interaction of several factors, such as increased permeability of intestinal barrier or increased secretion of gastrin, triggered by intense physical activity [Davis et al., 2003; McKenzie et al., 2010; Davis & Williamson, 2016].

NON-INVASIVE ASSESSMENT OF FECAL STRESS BIOMARKERS IN HUNTING DOGS DURING EXERCISE AND AT REST

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ABSTRACT

Intense exercise causes to organisms to have oxidative stress and inflammation at the gastrointestinal (GI) level. The reduction in intestinal blood flow and the exercise-linked thermal damage to the intestinal mucosa can cause intestinal barrier disruption, followed by an inflammatory response. Furthermore, the adaptation to exercise may affect the gut microbiota and the metabolome of the biofluids. The aim of the present research was to evaluate the presence of a GI derangement in hunting dogs through a non-invasive sampling as a consequence of a period of intense exercise in comparison with samples collected at rest. The study included nine dogs that underwent the same training regime for hunting wild boar. In order to counterbalance physiological variations, multiple-day replicates were collected and pooled at each experimental point for each dog. The samples were collected immediately at rest before the training (T0), after 60 days of training (T1), after 60 days of hunting wild boar (T2), and finally, at 60 days of rest after hunting (T3). A number of potential stress markers were evaluated: fecal cortisol metabolites (FCMs) as a major indicator of altered physiological states, immunoglobulin A (IgA) as an indicator of intestinal immune protection, and total antioxidant activity [total antioxidant capacity (TAC)]. Since stool samples contain exfoliated cells, we investigated also the presence of some transcripts involved in GI permeability [occludin (OCLN), protease-activated receptor-2 (PAR-2)] and in the inflammatory mechanism [interleukin (IL)-8, IL-6, IL-1b, tumor necrosis factor alpha (TNFα), calprotectin (CALP), heme oxygenase-1 (HO-1)]. Finally, the metabolome and the microbiota profiles were analyzed. No variation in FCM and IgA content and no differences in OCLN and CALP gene expression between rest and training were observed. On the contrary, an increase in PAR-2 and HO-1 transcripts, a reduction in total antioxidant activity, and a different profile of microbiota and metabolomics data were observed. Collectively, the data in the present study indicated that physical exercise in our model could be considered a mild stressor stimulus.

Key words: : dog, stool, gut microbiota, probiotic, IgA

INTRODUCTION

Intense exercise is known to exacerbate body stressors, such as oxidative stress and inflammation, the latter at both the muscular (1-3) and the gastrointestinal (GI) (4-6) levels. As a consequence, in performance sports, there is a high prevalence of GI problems both in humans, such as endurance runners (6-8), and in animals, such as horses (9, 10) or dogs (11). In a review paper by ter Steege et al. (12), several studies were cited that suggested that the key culprit behind GI symptoms during exercise was splanchnic hypoperfusion, which could lead to intestinal ischemia, thus subsequently damaging the intestinal epithelial cells and compromising the intestinal barrier function. Multiple studies involving humans have reported an exercise-induced increase in intestinal permeability (13). The tight junction (TJ) plays an important role in regulating the epithelial permeability by means of modifying the multiprotein complex [claudins and occludin (OCLN)] and/or promoting dysfunction to TJ regulatory proteins (i.e., zona-occludens) (14).

A downregulation of OCLN expression has been observed in different intestinal models, in which the permeability was strongly altered [i.e., inflammatory bowel disease (IBD), ulcerative colitis], and was downregulated (15, 16). Gut permeability is also influenced by protease-activated receptor-2 (PAR-2) expressed in the apical and basolateral membranes of intestinal epithelial cells (17). As described by a review (17), its activation induces an increase in permeability by means of impairment of the TJ functions, as shown in several epithelial and endothelial cell models (18-21). In different models including colitis and ischemia and reperfusion (I/R), PAR-2 transcription was upregulated in mouse, rodent, and horse models (21-23). Other markers of intestinal inflammation are calprotectin (CALP) and pro-inflammatory cytokines, which have been shown to be upregulated in IBD models (24-26).

Heme oxygenase-1 (HO-1) is an inducible cytoprotective stress-responsive protein induced by various stimuli, including oxidative stress I/R, heavy metals, and cytokines (27), the induction of which is usually associated with antioxidant, anti-apoptotic, and anti-inflammatory effects as reported by a review paper (28). In studies using murine experimental colitis models, HO-1 activity and expression were markedly increased, associated with the development of colitis, and the inhibition of HO activity potentiates colonic damage and inflammation (29, 30). Moreover, the relationship between physical exercise and increased HO-1 mRNA and protein expression/activity in different cells and tissues has already been demonstrated in rodents (31–34) as well as in humans (35, 36).

Cortisol is a well-known indicator of the stress response in the majority of mammals including dogs, with previous studies showing increased levels after exercise, such as agility work (37) and training in outdoor conditions (38, 39).

Many factors contribute to the maintenance of GI homeostasis. One of them is the secretion of immunoglobulin A (IgA), which coats the bacteria, favoring a tolerant, non- inflammatory

relationship with the host (40) and the homeostatic control of the intestinal redox environment (41). Previous papers have reported that exercise may affect the levels of IgA in mice (42) and cause oxidative stress in dogs (43).

Emerging research has suggested that intense exercise could also affect the gut microbiota. In particular, cross-sectional studies have shown an overall increase in biodiversity with some compositional alterations, mainly in mucin degraders, lactate utilizers, and short-chain fatty acid (SCFA) producers, in the intestinal microbial ecosystem of professional athletes (44). Several factors are likely to be involved, including changes in diet, hydration levels and metabolic flux, altered gut motility, and also impaired gut barrier function, as a result of exercise- induced heat stress and ischemia (44). Given the fundamental role of the gut microbiota in maintaining host metabolic and immunological homeostasis (45), its monitoring during periods of intense physical activity could help to elucidate the mechanisms underlying the microbial response to exercise and understand if and how these are related to host performance.

The metabolome of fluids, which is made up of the ensemble of low-weight organic molecules, results from a complex interaction between endogenous and exogenous host factors, including the gut microbiota. As such, it has been shown to give important information regarding the overall effects of exercise in both humans and animals, with specific reference to the inflammatory status. The fecal metabolome seems to be no exception, at least in rats (46).

The exfoliated enterocytes contained in feces have recently been used as a tool to investigate the impact of therapies and nutritional regimens on GI functions (47, 48). In fact, stool is easy to obtain and has already been used in quantifying intestinal gene expression profiles from exfoliated epithelial cells in neonates (49, 50), as well as under pathological conditions to detect candidate molecular biomarkers (51–53). Exercise induces multiple biochemical changes, which may affect the gene expression of the transcripts involved in the mitochondrial metabolism in muscle (54) and oxidative stress, as assessed non- invasively (i.e., in saliva) in avalanche military dogs (55).

The aim of the present research was to evaluate the presence of a GI derangement in hunting dogs through a non-invasive sampling as a consequence of a period of intense exercise in comparison with samples collected at rest. To reach this goal, we selected a number of potential stress markers in fecal samples, including cortisol metabolites [fecal cortisol metabolite (FCM)], transcripts involved in epithelial integrity and inflammatory mechanisms [cytokines: interleukin (IL)-8, IL-6, IL-1β, and tumor necrosis factor alpha (TNFα); OCLN; CALP; PAR-2; and HO-1], IgA, and total antioxidant capacity (TAC) levels. Furthermore, we decided to profile the fecal metabolome, by means of high-resolution proton magnetic resonance spectroscopy (^{1}H-NMR), and the microbiota, by 16S rRNA gene-based next-generation sequencing.

MATERIALS AND METHODS

Experimental Design and Exercise

Four experimental time points were set: T0, after 180 days of complete rest (rest before the training session, September); T1, after 60 days of training, 3 days a week, 3 h each day (November); T2, after 60 days of wild boar hunting three times a week, 5–6 h each day (January); and T3, after 60 days of complete rest (rest after hunting season, March) (Figure 1). The physical activity carried out during both the training (T1) and the hunting (T2) periods was similar and consisted of a first phase of identifying and locating prey and a subsequent chase phase. The duration of these phases, due to the nature of the hunting itself, varied and was therefore impossible to standardize. All the dogs equally and simultaneously participated in each training/hunting session. The training activity occurred on alternative days and was always conducted by the same person, the owner (not a professional trainer but an expert hunter fully aware of the goal of the research project), without any type of reinforcement.

Figure 1 Schedule of the experimental time points. T0, rest before the training; T1, 60 days of training; T2, 60 days of hunting season; T3, 60 days of rest after hunting

Animals

The exploratory study was carried out from September 2017 to March 2018 on nine hunting dogs. The dogs were of various ages (9.1 ± 5.0 years; mean ± SD), sexes (two unneutered males and seven spayed females), and breeds (three English Setter, three Segugio Italiano, two Dachsbacke, one Deutsch Kurzhaar) (Table 1). T0 body weight (BW) (19.3 ± 3.3 kg; mean ± SD) and the body condition score (BCS, calculated by using the 1– 9 score proposed by Royal Canine SAS) are reported in Table 1. BW and BCS were also determined at each experimental point. The dogs, owned by a single owner, were housed in individual boxes and fed, once a day, with a commercial diet (Eko Adult, Russo Mangimi SpA, NA, Italy): crude protein 22%, crude fats and oils 9%, crude fiber 4.6%, and crude ash 11.2%. The food was administered in relation to the weight of the dog and to physical activity, increasing the dose by about 15% in T1 and T2 with respect to the rest periods. All the dogs

underwent a physical examination by a veterinarian at the beginning of and during the trials. Only those who were clinically healthy were included in the study.

Dog	Breed	Gender	Age	BW (kg)	BCS 1–9
1	Segugio Italiano	SF	4	18	6
2	Dachsbracke	M	14	20	6
3	Deutsch Kurzhaar	SF	4	25	5
4	English Setter	M	13	22	6
5	Segugio Italiano	SF	7	18	4
6	Segugio Italiano	SF	16	20	5
7	Dachsbracke	SF	2	13	5
8	English Setter	SF	12	18	5
9	English Setter	SF	10	20	6

Table 1 Dogs included in the study.

M, unneutered male; SF, spayed female; BW, body weight; BCS, body condition score at T0 (rest before training).

Collection of the Fecal Sample

The samples were collected during the last week of each experimental period. In order to counterbalance the physiological fluctuations that occur within individuals, three samples for each time point were collected on different days. Specifically, at T1 and T2, the three samples were collected during the last week of physical activity on the day after the exercise session, while at T0 and T3, the three samples were collected on 3 consecutive days. The sample collection time was the same at each experimental point (after feeding in the late afternoon).

In agreement with the Italian law transposition of European Directive 2010/63 (DL 26/2014), the collection of fecal samples is not classified as a procedure, and it did not require any kind of authorization. This non-invasive sampling method was performed without any discomfort for the animals.

In total, 108 samples were collected: three for each dog at each of the four experimental times. The aforementioned three samples were pooled for the assays, leading to an overall sample number of 36 (one for each dog at each experimental time point).

Fresh fecal samples were collected by the owner within 1 h of defecation (late afternoon) and immediately stored at $-20^{\circ}C$ until analysis.

RNA Extraction and Reverse Transcription

Lyophilized fecal samples (Modulyo EF4 1044, Edwards) were weighed and resuspended with Dulbecco's phosphate-buffered saline (DPBS) (w/v; 100 mg/ml) by vortex mixing (3 min). RNA extraction was performed using PureZol RNA isolation reagent (BioRad, Bio-RAD Laboratories Inc., California, USA) and a NucleoSpin RNA II kit (Macherey Nagel, Duren, Germany). Briefly, 1 ml of PureZol RNA isolation reagent was added to 100 µl of each sample and vortex mixed (3 min). Two hundred microliters of chloroform was then added to the suspension and mixed well. After incubation at room temperature (10 min), the samples were centrifuged (12,000 g for 10 min), and the aqueous phase was recovered. One volume of ethanol was added, and the resulting solution was loaded onto a NucleoSpin RNA Column (light blue ring) (NucleoSpin RNA II kit, Macherey Nagel). The RNA was then purified according to the manufacturer's instructions and spectrophotometrically quantified (A260 nm) (DeNovix Inc., Wilmington, DE, USA). RNA (1 µg) was then reverse-transcribed to cDNA using an iScript cDNA Synthesis Kit (Bio-RAD), arriving at a final volume of 20 µl. An additional sample of canine intestinal biopsy, collected from the duodenum of a dog with IBD (derived from a diagnostic procedure, performed at DIMEVET, with the express consent of the owner; endoscopy code 9290, March 19, 2018, sample code 14873), underwent RNA extraction, reverse transcription, and subsequent analysis (quantitative real-time PCR assay) as a positive control of inflammatory gene expression.

Quantitative Real-Time PCR

Real-time quantitative PCR was carried out using a CFX 96 Real Time System (Bio-RAD) and SsoAdvanced™ Universal SYBR®R Green Supermix (Bio-RAD). All the samples were analyzed in duplicate (10 µl/well), and the qPCR assays were carried out for different references [glyceraldehyde-3-phosphate dehydrogenase (GAPDH), TATA-box binding protein (TBP), tight junction protein 1 (TJP1), ribosomal protein L32 (RPL32), succinate dehydrogenase (SDHA), and interest genes (Il-8, IL-1β, IL-6, TNFα, OCLN, CALP, PAR-2, HO-1]. Primer sequences are reported in Table 2. Real-time efficiency was evaluated by amplification of a standardized amount of cDNA, starting from 150 ng with subsequent 5-fold dilutions (75, 15, 3, 0.6, and 0.12 ng), derived from both fecal sample–derived and intestinal cDNA (duodenal biopsy). The specificity of the amplified PCR products was verified by analysis of the melting curve and agarose gel electrophoresis. The relative gene expression was calculated as the fold increase using the 2^{-OOCt} method (58) in relation to T0 (OOCt = OCt T1 or T2 or T3 group –OCt T0).

Gene		Primer sequence (5′->3′)	PCR (bp)	AN	References
HO-1	F	GCCAGTGCCACGAAGTTC	164	NM_001194969	Present study
	R	TCCTCAGTGTCCTTGCTCAG			
CALP	F	ACCATGCTGACGGAACTGGAGAG	244	NM_001146144	Present study
	R	CCACGCCCACCTTTATCACCAATATG			
OCLN	F	CAGAGTCTTCCTATAAATCAAC	196	NM_001003195.1	Present study
	R	GTGTAGTCTGTCTCATAGTG			
PAR-2	F	TGAAGATCGCCTACCACATCCG	137	AB_458680	(56)
	R	CCAATACCGTTGCACACTGA			
IL-8	F	CTTCCAAGCTGGCTGTTGCTC	173	NM_001003200	(56)
	R	TGGGCCACTGTCAATCACTCTC			
IL-1β	F	GCTGCTGCCAAGACCTGAAC	112	XM_005630074	Present study
	R	GCTACAATGACTGACACGAAATGC			
TNFα	F	CCCAAGTGACAAGCCAGTAGCTC	146	NM_001003244	(56)
	R	ACAACCCATCTGACGGCACTATC			
IL-6	F	AAAGAGCAAGGTAAAGAATCAGGATG	126	NM_001003301	Present study
	R	CGCAGGATGAGGTGAATTGTTG			
GAPDH	F	TGTCCCCACCCCCAATGTATC	100	NM_001003142	(57)
	R	CTCCGATGCCTGCTTCACTACCTT			
TBP	F	CTATTTCTTGGTGTGCATGAG G	96	XM849432	(56)
	R	CCT CGG CATTCAGTCTTTTC			
TJP1	F	GCTGTGGAAGAAGATGAAGATG	175	NM_001003140	Present study
	R	CTCGGCAGACCTTGAAGTAG			
RPL32	F	GGCACCAGTCAGACCGATATG	209	NM_001252169	Present study
	R	GCACATCAGCAGCACTTCAAG			
SDHA	F	CGCATAAGAGCCAAGAAC	194	XM535807	Present study
	R	CCTTCCGTAATGAGACAAC			

Table 2 List of primer pairs, amplicon size (bp), and accession number (AN) in the NCBI (National Center of Biotechnology Information) database.

HO-1, heme oxygenase-1; CALP, calprotectin; OCLN, occludin; PAR-2, protease-activated receptor-2; IL, interleukin; TNFα, tumor necrosis factor alpha; GAPDH, glyceraldehyde-3- phosphate dehydrogenase; TBP, TATA-box binding protein; TJP1, tight junction protein 1; RPL32, ribosomal protein L32; SDHA, succinate dehydrogenase

Fecal Cortisol Metabolites Determination

Extraction from the feces was performed as previously described (59). Briefly, a methanol:water (v/v 4:1) solution was added to the feces in capped glass tube vials. The vials were then vortex mixed for 30 min using a multitube pulsing vortexer. Following centrifugation (1,500 g for 15 min), ethylic ether and $NaHCO_3$ (5%) were added to 1 ml of supernatant. This preparation was then vortex mixed for 1 min on a multitube pulsing vortexer and centrifuged for 5 min (1,500 g). The ether portion was then separated and evaporated to dryness under an air-stream suction hood at 37°C; finally, the dry residue was dissolved into phosphate-buffered saline (PBS) 0.05 M, pH 7.5. Radio immunological assay (RIA) was carried out according to Tamanini et al. (60). Analysis was carried out in duplicate. The parameters for analysis validation were: sensitivity 0.23 pg/mg; intra-assay variability 6.4%; inter-assay variability 9.7%; and specificity (%) of cortisol 100, corticosterone 9.5, 11α-hydroxy-progesterone 8.3, cortisone 5.3, 11α-deoxycortisol 5.0, progesterone 0.6 deoxycorticosterone

0.5, 20α-dihydrocortisone 0.4, testosterone 0.3, aldosterone 0.1, and dehydroepiandrosterone, 5α-pregnenolone, 17β-estradiol, and cholesterol <0.0001.

Determination of Total IgAs and TAC

The IgA extraction was performed essentially as reported by Peters et al. (61).

Briefly, the lyophilized fecal samples were placed in 1 ml (w/v; 100 mg/ml) of extraction buffer (PBS containing 0.5% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) and a protease inhibitor cocktail (Sigma, 1 tablet/25 ml), and after the addition of three 3 mm glass beads, the samples were homogenized for 1 min with TissueLyser (50 Hz) (QIAGEN, Hilden, Germany). The homogenates were then centrifuged (1,500 g for 15 min), and the recovered supernatants were additionally centrifuged (15,000 g for 20 min). The supernatants were frozen at −20°C until analysis.

The IgA level was measured by a specific enzyme-linked immunosorbent assay (ELISA) kit (Dog IgA ELISA Quantitation Set, Bethyl Laboratories Inc., Montgomery, TX, USA). The analyses were carried out in duplicate. The parameters for analysis validation were: intra-assay variability 2.1% and inter-assay variability 12.8%. After checking the parallelism (R^2 = 0.9849, unpublished data), we diluted the sample 1:75,000 and carried out the assay according to the manufacturer's instructions.

The TAC level was assayed by using an Antioxidant Assay Kit (item no. 709001; Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions and expressed as a Trolox equivalent.

Metabolomics

The fecal samples were prepared for ^{1}H-NMR analysis by vortex mixing for 5 min (80 mg of stool with 1 ml of deionized water). The mixtures were then centrifuged for 15 min at 18,630 g and 4°C. The supernatants (700 µl) were added to a D_2O solution of 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP) 10 mM and NaN_3 2 mM, set at pH 7.00 ± 0.02 with 1 M potassium phosphate buffer. Before analysis, the samples were centrifuged again at the above conditions.

The ^{1}H-NMR spectra were recorded at 298 K using an AVANCE III spectrometer (Bruker, Milan, Italy) operating at a frequency of 600.13 MHz. In accord with Ventrella et al. (62), the signals from broad resonances originating from large molecules were suppressed by a Carr–Purcell–Meiboom–Gill (CPMG) filter composed by 400 echoes with a τ of 400 µs and a 180° pulse of 24 µs, for a total filter of 330 ms. The HOD residual signal was suppressed by means of pre-saturation. Each spectrum was acquired by summing up 256 transients using 32 K data points over a 7,184 Hz spectral window, with an acquisition time of 2.28 s. To apply NMR as a quantitative technique (63), the recycle delay was set to 5 s, taking into consideration the relaxation time of the protons under investigation. ^{1}H-NMR spectra were baseline-adjusted by means of the peak detection according to the "rolling ball"

principle (64) implemented in the baseline R package (65). A linear correction was then applied to each spectrum, so as to make the points pertaining to the baseline randomly spread around zero. Spectra have been horizontally aligned by employing the signal of TSP as a reference. The differences in water and fiber content among the samples were taken into consideration using probabilistic quotient normalization (66), applied to the entire spectra array.

The signals were assigned by comparing their chemical shift and multiplicity with the Human Metabolome Database (67) and Chenomx software data bank (Chenomx Inc., Canada, version 8.1).

Microbial DNA Extraction and 16S rRNA Gene Sequencing

Microbial DNA was extracted from the fecal samples using the DNeasy Blood & Tissue kit (QIAGEN), with a modified protocol as previously described (68). Briefly, 250 mg of feces were resuspended in 1 ml of lysis buffer (500 mM NaCl, 50 mM Tris- HCl pH 8, 50 mM EDTA, 4% SDS). Four 3 mm glass beads and 0.5 g of 0.1 mm zirconia beads (BioSpec Products, Bartlesville, OK) were added to the fecal samples and homogenized with three bead-beating steps using the FastPrep instrument (MP Biomedicals, Irvine, CA) at 5.5 movements/s for 1 min, keeping the samples on ice for 5 min after each treatment. The samples were heated at 95°C for 15 min and centrifuged for 5 min at 13,000 g to pellet stool particles. The supernatants were collected, and 260 µl of 10 M ammonium acetate was added; the samples were then incubated on ice for 5 min and then centrifuged for 10 min at 13,000 g. One volume of isopropanol was added, and the supernatants were incubated on ice for 30 min. The nucleic acids were collected by centrifugation for 15 min at 13,000 g and washed with 70% ethanol. The pellets were then resuspended in 100 µl of Tris-EDTA (TE) buffer and treated with 2 µl of DNase-free RNase (10 mg/ml) for 15 min at 37°C. Protein removal and DNA purification using QIAamp Mini Spin columns (QIAGEN) were carried out according to the kit protocol. The DNA extracted was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). For each sample, the V3–V4 region of the 16S rRNA gene was sequenced as previously reported (69). Briefly, the DNA was amplified using the S-D-Bact-0341-b-S-17/S-D-Bact-0785- a-A-21 primers (70) with Illumina overhang adapter sequences. PCR products of ∼460 bp were purified using a magnetic bead- based system (Agencourt AMPure XP; Beckman Coulter, Brea, CA), indexed by limited-cycle PCR using Nextera technology, and were additionally purified using Agencourt AMPure XP magnetic beads. Indexed libraries were pooled at an equimolar concentration, denatured, and diluted to 6 pmol/L before loading onto the MiSeq flow cell. Sequencing was carried out on an Illumina MiSeq platform using a 2 × 250 bp paired-end protocol, according to the manufacturer's instructions (Illumina, San Diego, CA). Sequencing reads were deposited in the National Center for Biotechnology Information Sequence Read Archive (NCBI SRA; BioProject ID PRJNA 589580).

Bioinformatics and Statistical Analysis

Statistical analysis was carried out in R computational language (71). Differences among sampling points were assessed using the analysis of variance (ANOVA) test for repeated measures (*P*-value < 0.05 was considered statistically significant). Robust principal component analysis (rPCA) models were calculated as described by Hubert et al. (72), namely, by accepting an alpha value of 0.75. Differences in the mRNA data were evaluated using one-way ANOVA (*P*-value < 0.05 was considered statistically significant).

As for the gut microbiota analysis, raw sequences were processed using a pipeline combining PANDAseq (73) and QIIME 2 (74). High-quality reads were filtered and clustered into amplicon sequence variants (ASVs) at 99% similarity by means of an open-reference strategy carried out using dada2 (75). Taxonomy was assigned using the vsearch classifier (76) and the Greengenes database as a reference (release May 2013). Alpha rarefaction was carried out using Faith's phylogenetic index and the number of observed ASVs, while beta diversity was estimated by computing weighted and unweighted UniFrac distances. All the statistical analyses were carried out using R (version 3.1.3) and the packages vegan and made4. UniFrac distances were used for the principal coordinate analysis (PCoA), and the significance of data separation was tested using a permutation test with pseudo-*F* ratios (function adonis of vegan) and the ANOSIM test. The Wilcoxon test for paired data was used to assess significant differences in alpha diversity and taxon relative abundance between groups, while the Kruskal–Wallis test was used for multiple comparisons. A *P*-value < 0.05 was considered statistically significant.

RESULTS

Animals

In Figure 2, we report the variation in BW of the dogs during the trial.

The physical activity induced a statistically significant decrease of BW after 60 days of hunting season (T2, 16.9 ± 3.4) with respect to the rest periods (T0, 19.3 ± 3.3, and T3, 18.8 ± 3.2) ($P = 0.017$, repeated measures ANOVA, Tukey's multiple comparison test, $P < 0.05$). On the contrary, the training period did not significantly influence the BW (T1, 18.7 ± 3.6) (repeated measures ANOVA, Tukey's multiple comparison test, $P < 0.05$). The percentages of BW reduction at T1, T2, and T3 with respect to T0 were 3.2, 12.7, and 2.9%, respectively. The BCSs of the dogs recorded during the trial were (median, min–max): T0 (4, 5, 5, 6); T1 (4, 5, 5, 6); T2 (3, 4, 4); and T3 (4, 5, 5, 6). Similarly to BW, only T2 (60 days after hunting season) was statistically different from rest periods (T0 and T3) and the period after 60 days of training (T1) (repeated measures ANOVA, Friedman test, Dunn's multiple comparison test, $P < 0.05$).

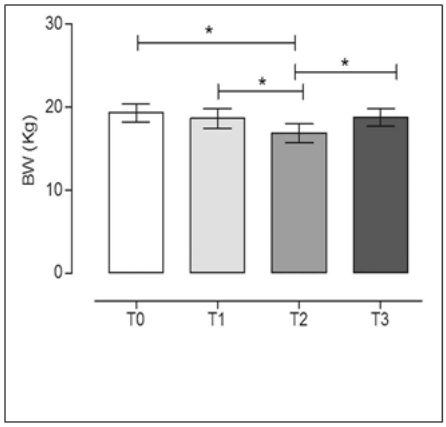

Figure 2: Body weight (BW) of dogs at the different time points. The physical activity induced a statistically significant decrease in BW after 60 days of hunting season (T2) (mean ± SEM) (P = 0.017). *Indicates P < 0.05 (repeated measures ANOVA, Tukey's multiple comparison test, P < 0.05).

Real-Time Quantitative Reverse Transcription PCR for PAR-2, HO1, CALP, OCLN, IL-8, IL-1β, IL-6, and TNFα

RNA was extracted from all the samples with a yield of 336.35 ± 147.8 ng/10 mg dry feces. Of the reference genes analyzed, only GAPDH was always detectable; therefore, it was used as a reliable internal reference for qPCR normalization. To evaluate the matrix effect, we determined qPCR efficiency for GAPDH in the stool and tissue samples. The results showed that the efficiency was similar in both samples (97 and 91.7%, respectively) (Figure 3), indicating that RNA isolated from feces did not contain particular PCR inhibitors.

The presence and specificity of the PCR products were verified using melting curve analysis and agarose gel electrophoresis. The transcripts of GAPDH, HO-1, CALP, OCLN, and PAR-2 were detectable in the majority of the samples analyzed (GAPDH 33/36, HO-1 29/36, PAR-2 27/36, CALP 21/36, OCLN 26/36), although with a huge variability regarding the range of gene expression both between the dogs and regarding the time points.

The expression levels of OCLN and CALP did not show significant differences among groups (P = 0.6338 and P = 0.1704, respectively) (one-way ANOVA, Tukey's multiple comparison test, P < 0.05, Figure 4). On the contrary, a statistically significant increase was observed at T2 (after 60 days of hunting season) for PAR-2 and HO-1 as compared to T0 (P = 0.042 and P = 0.028, respectively) (one-way ANOVA, Tukey's multiple comparison test, P<0.05, Figure 4). Very low or undetectable expression levels were observed for the genes encoding the cytokines (IL-8, IL-1β, IL-6, TNFα) (very low 7/36, undetectable 29/36) and for the other reference genes (TPB, TJP1, RPL32, SDHA) (very low 8/36, undetectable level 28/36).

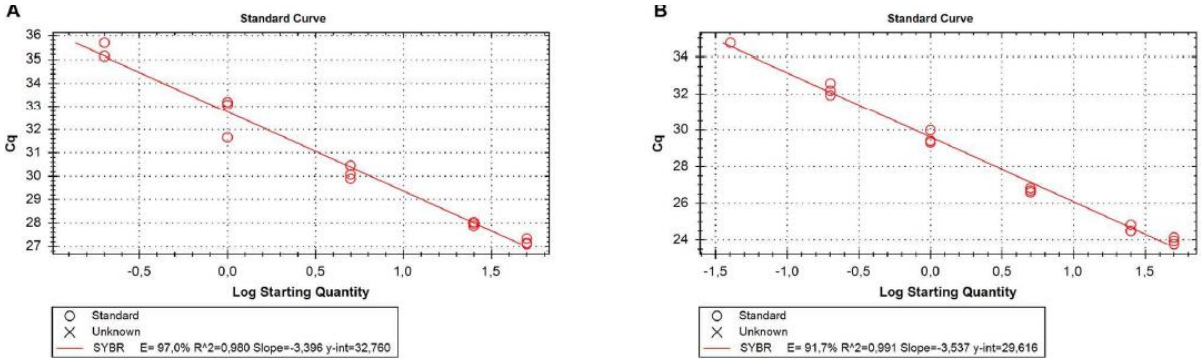

Figure 3 Quantitative real time PCR (qRT-PCR) efficiency for the reference gene [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)]. Five different 5-fold dilutions of the stool (A) or tissue (B) samples were assayed. Cq, cycle quantification; E, efficiency

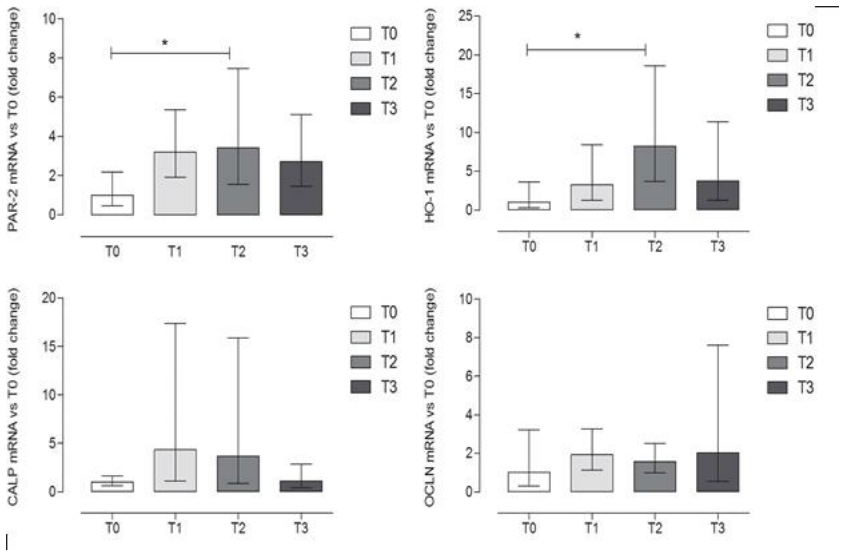

Figure 4 Gene expression of protease-activated receptor-2 (PAR-2), heme oxygenase-1 (HO-1), calprotectin (CALP), and occludin (OCLN) evaluated by qRT-PCR, at the different time points. Relative gene expression of PAR-2, HO-1, CALP, and OCLN in the fecal samples of dogs at rest before training (T0), after 60 days of training (T1), after 60 days of hunting season (T2), and at 60 days of rest after hunting (T3). The mRNA data are expressed as fold change with respect to T0.

*Indicates $P < 0.05$ (one-way ANOVA, $P < 0.05$, post-hoc Tukey's test). P-values: $P = 0.042$ for PAR-2; $P = 0.028$ for HO-1; $P = 0.1704$ for CALP; $P = 0.6338$ for OCLN. Error bars represent the range of gene expression.

FCM Determination

No statistically significant differences were observed in FCM content during the trial (P = 0.270) (repeated measures ANOVA, $P < 0.05$). The concentration of FCMs at T0 was 0.31 ± 0.03 pg/mg feces, while at T1, the level was 0.63 ± 0.29 pg/mg feces (Figure 5).

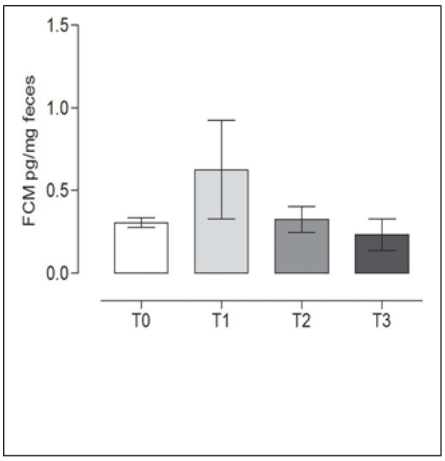

Figure 5 Fecal cortisol metabolites (FCMs) at the different time points. The concentration of cortisol metabolites (mean ± SEM) in the fecal samples of the dogs at rest before training (T0), after 60 days of training (T1), after 60 days of hunting season (T2), and at 60 days of rest after hunting (T3). No statistically significant differences (P = 0.2760) were observed (repeated measures ANOVA, Tukey's multiple comparison test, $P < 0.05$).

Determination of Total IgA in Stools

The IgA content in the canine fecal samples at the different time points is reported in Figure 6. No statistically significant differences among the groups were observed (P = 0.065) (repeated measures ANOVA, $P < 0.05$).

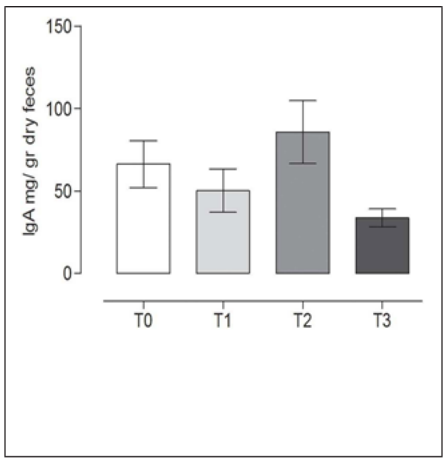

Figure 6 Immunoglobulin A (IgA) concentrations in the stool at the different time points. The IgA concentrations (mean ± SEM) in the fecal samples of dogs at rest before training (T0), after 60 days of training (T1), after 60 days of hunting season (T2), and at 60 days of rest after hunting (T3). No statistically significant differences ($P = 0.065$) were observed (repeated measures ANOVA, Tukey's multiple comparison test, $P < 0.05$).

Determination of Total Antioxidant Activity

TAC showed a slight variation during the study, with a statistically significant difference between T1 (19.82 ± 0.79, mean ± SD) (after 60 days of training) and the rest after the hunting season, T3 (22.89 ± 0.89, mean ± SD) (60 days of rest after hunting) (P = 0.0213) (repeated measures ANOVA, P < 0.05, Figure 7).

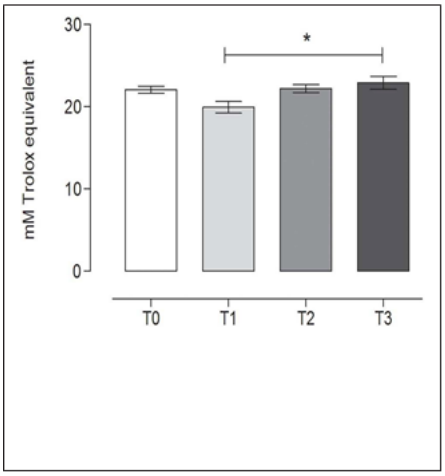

Figure 7 Total antioxidant capacity (TAC) in the fecal samples at the different time points. The TAC value (mean ± SEM) in the fecal sample at rest before training (T0, 22.74 ± 0.46), after 60 days of training (T1, 19.82 ± 0.79), after 60 days of hunting season (T2, 22.16 ± 0.56), and at 60 days of rest after hunting (T3, 22.89 ± 0.89). The TAC was significantly lower at T1 than at T3 ($P = 0.0213$). *Indicates $P < 0.05$ (repeated measures ANOVA, Tukey's multiple comparison test, $P < 0.05$).

Metabolomics of the Feces

In order to explore the changes in the fecal metabolome of the dogs involved in the study, the ^{1}H-NMR spectra were registered. Seventy-three molecules could be quantified. Seventeen molecules, reported in **Table 3**, showed a concentration that differed among the time points investigated.

To observe the overall trends driving the changes that these molecules underwent, their concentrations were used as a basis for an rPCA model, as depicted in **Figure 8**. Along PC1 of its score plot (**Figure 8A**), representing as much as 62.7% of the entire sample's variability explained by the PCA, the metabolomes of the dogs at T0 and T1 were characterized by the highest and the lowest scores, respectively, while the fecal metabolomes of the dogs at T2 and T3 appeared in intermediate positions. Specifically, the samples at T0, T1, and T2 appeared to be significantly separated from one other, while the metabolome at T3 was not distinguishable from that at T1 or T2. **Figure 8C** is a pictorial representation that highlights how all the molecules that have changed significantly over time tended to have the lowest concentrations at T0. The molecules mainly responsible for grouping the samples in this respect were proline, galacturonate and formate, 1,3-dihydroxyacetone, uridine, malate, 3-hydroxyphenylacetate, methylamine, and fucose.

	T0	T1	T2	T3	*P*-value
Formate	$4.95 \times 10^{-5} \pm 1.47 \times 10^{-4\ b}$	$2.32 \times 10^{-4} \pm 5.96 \times 10^{-5\ a}$	$2.37 \times 10^{-4} \pm 4.25 \times 10^{-5\ a}$	$2.82 \times 10^{-4} \pm 1.76 \times 10^{-4\ a}$	6.72E-04
Uridine	$1.85 \times 10^{-4} \pm 8.83 \times 10^{-5\ b}$	$3.65 \times 10^{-4} \pm 1.14 \times 10^{-4\ a}$	$3.89 \times 10^{-4} \pm 1.30 \times 10^{-4\ a}$	$3.98 \times 10^{-4} \pm 1.71 \times 10^{-4\ a}$	3.89E-04
3-Hydroxyphenylacetate	$2.63 \times 10^{-4} \pm 4.25 \times 10^{-4\ b}$	$1.69 \times 10^{-3} \pm 9.38 \times 10^{-4\ a}$	$1.71 \times 10^{-3} \pm 8.45 \times 10^{-4\ a}$	$1.26 \times 10^{-3} \pm 7.45 \times 10^{-4\ ab}$	1.13E-05
Galactose	$3.02 \times 10^{-5} \pm 9.33 \times 10^{-5\ c}$	$5.16 \times 10^{-4} \pm 3.48 \times 10^{-4\ b}$	$2.78 \times 10^{-4} \pm 1.07 \times 10^{-4\ a}$	$3.39 \times 10^{-4} \pm 2.84 \times 10^{-4\ ab}$	4.16E-06
Arabinose	$8.57 \times 10^{-4} \pm 4.92 \times 10^{-4\ b}$	$2.99 \times 10^{-3} \pm 1.16 \times 10^{-3\ a}$	$2.50 \times 10^{-3} \pm 8.47 \times 10^{-4\ a}$	$2.05 \times 10^{-3} \pm 1.11 \times 10^{-3\ a}$	1.13E-05
Fucose	$4.52 \times 10^{-5} \pm 2.02 \times 10^{-4\ b}$	$5.88 \times 10^{-4} \pm 2.30 \times 10^{-4\ a}$	$4.72 \times 10^{-4} \pm 3.19 \times 10^{-4\ a}$	$4.36 \times 10^{-4} \pm 1.89 \times 10^{-4\ a}$	6.88E-05
1,3-Dihydroxyacetone	$1.23 \times 10^{-5} \pm 2.44 \times 10^{-5\ b}$	$1.39 \times 10^{-4} \pm 1.24 \times 10^{-4\ a}$	$9.51 \times 10^{-5} \pm 7.63 \times 10^{-5\ a}$	$9.42 \times 10^{-5} \pm 8.98 \times 10^{-5\ a}$	3.35E-04
Galacturonate	$6.51 \times 10^{-5} \pm 9.02 \times 10^{-5\ c}$	$1.94 \times 10^{-4} \pm 8.84 \times 10^{-5\ b}$	$1.08 \times 10^{-4} \pm 6.96 \times 10^{-5\ abc}$	$1.35 \times 10^{-4} \pm 5.67 \times 10^{-5\ a}$	1.30E-05
Malate	$7.92 \times 10^{-4} \pm 6.07 \times 10^{-4\ b}$	$1.83 \times 10^{-3} \pm 9.29 \times 10^{-4\ a}$	$1.46 \times 10^{-3} \pm 1.01 \times 10^{-3\ ab}$	$2.32 \times 10^{-3} \pm 1.93 \times 10^{-3\ a}$	3.48E-02
Threonine	$8.07 \times 10^{-4} \pm 5.81 \times 10^{-4\ b}$	$2.14 \times 10^{-3} \pm 6.66 \times 10^{-4\ a}$	$1.91 \times 10^{-3} \pm 3.42 \times 10^{-4\ a}$	$1.99 \times 10^{-3} \pm 5.16 \times 10^{-4\ a}$	1.17E-03
Glycine	$2.07 \times 10^{-3} \pm 4.68 \times 10^{-4\ b}$	$4.82 \times 10^{-3} \pm 4.85 \times 10^{-3\ ab}$	$2.80 \times 10^{-3} \pm 7.04 \times 10^{-4\ ab}$	$3.33 \times 10^{-3} \pm 9.62 \times 10^{-4\ a}$	1.70E-03
Methanol	$2.37 \times 10^{-4} \pm 2.06 \times 10^{-4\ b}$	$6.02 \times 10^{-4} \pm 2.77 \times 10^{-4\ a}$	$4.48 \times 10^{-4} \pm 1.29 \times 10^{-4\ ab}$	$4.98 \times 10^{-4} \pm 2.82 \times 10^{-4\ ab}$	2.02E-02
Proline	$2.39 \times 10^{-4} \pm 1.31 \times 10^{-4\ b}$	$6.64 \times 10^{-4} \pm 1.49 \times 10^{-4\ a}$	$6.58 \times 10^{-4} \pm 3.27 \times 10^{-4\ ab}$	$6.57 \times 10^{-4} \pm 1.36 \times 10^{-4\ a}$	2.10E-05
Trimethylamine (TMA)	$3.99 \times 10^{-4} \pm 2.36 \times 10^{-4\ ab}$	$2.65 \times 10^{-4} \pm 1.44 \times 10^{-4\ b}$	$4.04 \times 10^{-4} \pm 1.27 \times 10^{-4\ a}$	$4.44 \times 10^{-4} \pm 3.00 \times 10^{-4\ ab}$	4.53E-02
Homocystine	$3.17 \times 10^{-4} \pm 5.05 \times 10^{-4\ b}$	$2.42 \times 10^{-3} \pm 1.56 \times 10^{-3\ a}$	$2.17 \times 10^{-3} \pm 1.41 \times 10^{-3\ a}$	$1.52 \times 10^{-3} \pm 8.90 \times 10^{-4\ a}$	6.01E-05
Methylamine	$1.84 \times 10^{-4} \pm 8.64 \times 10^{-5\ b}$	$4.20 \times 10^{-4} \pm 2.34 \times 10^{-4\ ab}$	$3.81 \times 10^{-4} \pm 1.08 \times 10^{-4\ a}$	$3.02 \times 10^{-4} \pm 1.21 \times 10^{-4\ b}$	1.13E-03
Valine	$1.80 \times 10^{-3} \pm 7.53 \times 10^{-4\ b}$	$2.51 \times 10^{-3} \pm 8.29 \times 10^{-4\ ab}$	$2.66 \times 10^{-3} \pm 5.34 \times 10^{-4\ ab}$	$2.85 \times 10^{-3} \pm 9.57 \times 10^{-4\ a}$	4.42E-02

Table 3: Temporal dynamics of the fecal metabolome of hunting dogs following physical activity.

Concentration (mmol/g, mean ± SD) of the molecules significantly differed among groups (Repeated Measure ANOVA, $P < 0.05$).
*For each molecule, different superscript letters identify significant differences among the groups ($P < 0.05$). For each molecule P value was reported.

The Structure and the Variations of the Gut Microbiota of Hunting Dogs as Related to Physical Activity

The 16S rRNA gene-based next-generation sequencing yielded a total of 1,390,231 high-quality reads, with an average of 39,720 ± 12,005 sequences per sample, binned in 1,460 ASVs at 99% similarity.

The PCoA of inter-sample variation based on weighted and unweighted UniFrac distances showed significant separation among the study groups ($P < 0.03$, permutation test with pseudo-F ratios; $P \leq 0.02$, ANOSIM) (**Figure 9A**). In particular, according to both the adonis and the ANOSIM statistics applied to the unweighted UniFrac-based ordination, the samples at T1 and T2 segregated from those at T0 ($P < 0.005$), while the T3 samples occupied an intermediate position (**Table S1**). No significant differences were found in alpha diversity, even though Faith's phylogenetic index showed an increasing trend over time (**Figure 9B**).

In line with the literature available regarding the gut microbiota of healthy dogs (77, 78), the fecal microbial profiles at the baseline were dominated by the phylum Firmicutes (relative abundance, mean ± SEM, 69.6 ± 8.1%), with Bacteroidetes (12.0 ± 5.2%), Actinobacteria (6.7 ± 3.1%), Proteobacteria (6.0 ± 2.4%), and Fusobacteria (5.5 ± 4.1%) as minor components. Similar proportions were observed during training, hunting, and the subsequent rest period, except for a reduction in the relative abundance of Proteobacteria after training ($P < 0.01$, Wilcoxon test). *Clostridiaceae*, *Erysipelotrichaceae*, and *Lactobacillaceae* were the major families of the baseline microbiota (relative abundance > 10%). Following training, an increase in the relative abundance of *Streptococcaceae* and *Enterococcaceae* was observed ($P < 0.05$). Such an increase persisted for *Streptococcaceae* ($P = 0.008$) until the rest period after hunting, while for *Enterococcaceae,* relative abundance

values comparable to the baseline were restored (**Figure 10**). In contrast, diminished proportions were observed for *Prevotellaceae* and *Ruminococcaceae* after training ($P < 0.04$).

Consistent with the above results, the main discriminant genera were *Streptococcus* and *Enterococcus*, the relative abundance of which was significantly greater at T1 than at T0 ($P < 0.04$), and *Prevotella*, the proportions of which decreased after training ($P \leq 0.03$) (**Figure 10**).

Although not significant, a decreasing trend was observed for *Faecalibacterium* and *Bacteroides* after physical activity (i.e., training and hunting) compared to both rest periods (i.e., before the training and after the hunting season). At rest, the baseline relative abundance of *Enterococcus* was restored, whereas the proportions of *Streptococcus* remained higher than the baseline ($P = 0.008$).

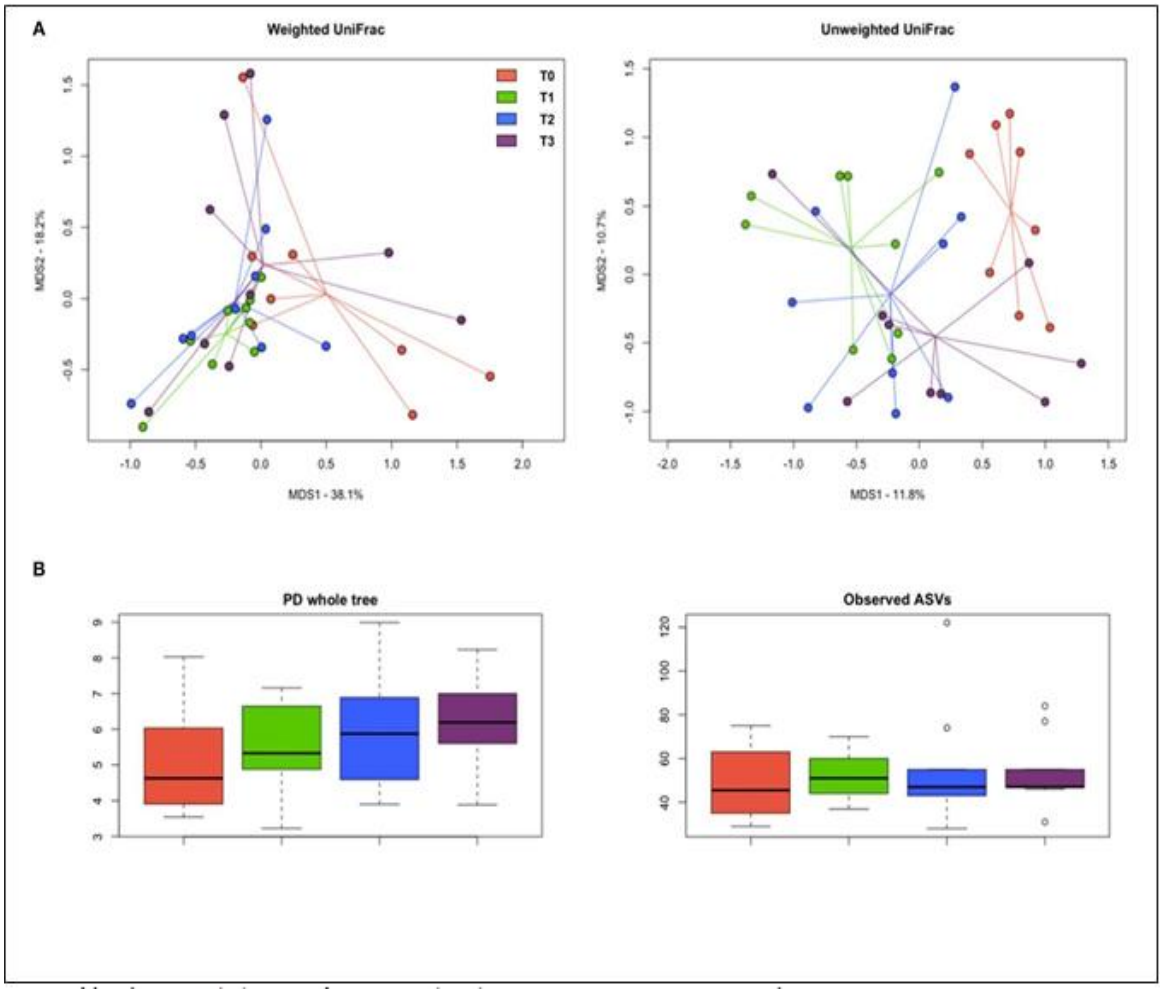

Figure 9 Diversity of the gut microbiome in hunting dogs. (A) Principal coordinate analysis (PCoA) plots showing the beta diversity of the gut microbial communities of the study groups (rest before training, T0; after 60 days of training, T1; after 60 days of hunting season, T2; at 60 days of rest after hunting, T3), based on weighted and unweighted UniFrac distances. A significant separation among groups was found ($P < 0.03$, permutation test with pseudo-*F* ratios; $P \leq 0.02$, ANOSIM). (B) Box plots showing alpha diversity, computed with Faith's phylogenetic index (PD whole tree) and the number of observed amplicon sequence variants (ASVs).

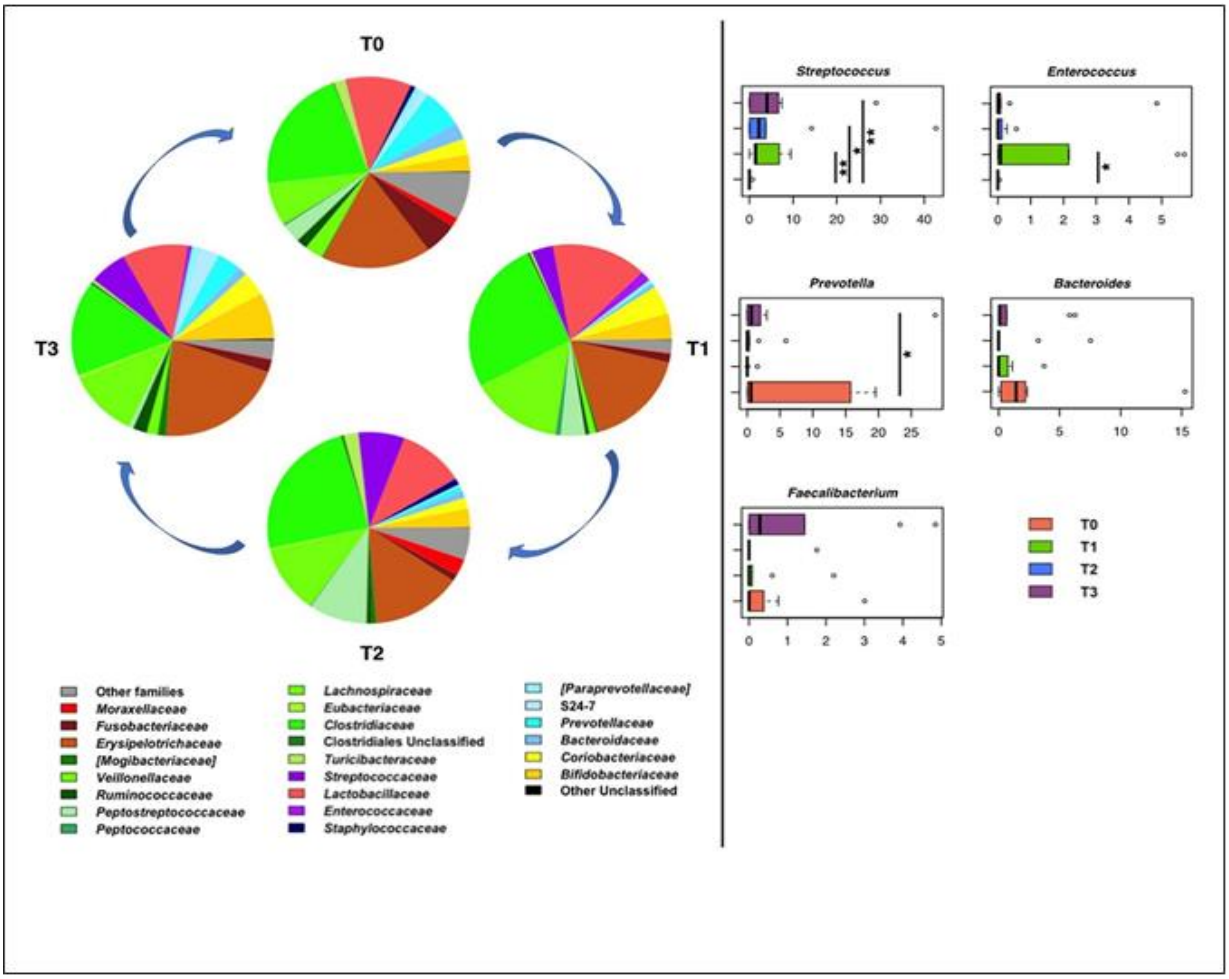

Figure 10 Temporal dynamics of the gut microbiome in hunting dogs following physical activity. Left, pie charts representing the average values of family-level relative abundances at each time point (rest before training, T0; after 60 days of training, T1; after 60 days of hunting season, T2; at 60 days of rest after hunting, T3). Right, box plots showing the distribution of the relative abundances of significantly enriched or depleted bacterial genera over time. **P* < 0.04; ***P* < 0.001 (Wilcoxon test). For *Bacteroides* and *Faecalibacterium*, only a decreasing trend was observed.

DISCUSSION

Inx this study, we evaluated the fluctuations of different stress markers in fecal samples of hunting dogs during physical activity and at rest. The main limitation for such studies lies within the difficulty in standardizing the training protocol (wild boar hunting) and the management of privately owned animals (diet, housing, treatments, etc.). In order to try and overcome this problem, we chose a group of dogs owned by the same person, in this case, one of the animal technicians of the Veterinary Department. He is indeed routinely involved in the husbandry and care of animals for both clinical and experimental purposes, and he was fully aware of the goals of the experiment and of the potential biases imputable to variations in the management of animals enrolled in such trials.

This choice has added a limiting factor to the study, which, being exploratory, included a low number of dogs of different breeds and ages, variables known to potentially influence the results (79); nonetheless, the study design allowed for a high level of standardization in terms of dogs' management, making for reliable results despite the relatively low sample size.

Typically, performance dogs are kept at 4–5/9 BCS due to the great chance of body condition loss during endurance activity, and the diet was calculated to support this condition. Weight loss is known to be related with some of the parameters measured in this research, for instance, microbiota (80), metabolomics profile (81), and cortisol (82). The expected weight loss observed during the trial has to be interpreted as a direct consequence of physical activity and not of a caloric restriction, so its potential effects on the measured parameters could be considered as a direct consequence of the physical activity.

In the model used in the present study, fecal cortisol, a well-known marker of stress in dogs, did not show any difference across the time points. In previous investigations, increases in cortisol concentration after sustained exercise had been observed in horses and humans, while the data regarding dogs were contradictory. In fact, some papers have reported increased levels of cortisol (83, 84), while others agreed with the present study in reporting no significant changes (85, 86). In particular, Pastore et al. (37) and Ando et al. (39) reported that cortisol increased right after exercise but returned to baseline levels shortly after, suggesting a mild transient stress. Similarly, in the present study, cortisol showed a transient non-significant increase during the first phase of activity (60 days of training, T1) only. Moreover, all samples were collected during a short-day period (autumn–winter), avoiding the reported interference of photoperiod on the cortisol concentrations (87).

Intestinal IgA secretion is considered to be an important indicator of mucosal immunity. Similar to cortisol, the literature regarding the effect of exercise on IgA secretion is contradictory, reporting either an increase or a decrease in intestinal IgA in mice (42, 88, 89). Based on the present data, training might not influence IgA concentration, confirming that exercise does not drastically alter canine intestinal immune homeostasis.

A previous paper indicated an increase in oxidative stress in hunting dogs after exercise (43): in accordance with this paper, the data in the present study also showed a significant and transient reduction in TAC during T1 (60 days of training), in relation to T3 (60 days of rest after hunting), suggesting an increase in oxidative stress following the resumption of physical activity.

In agreement with the human results, in the present study, we were able to detect different biomarkers' transcripts in dog stool samples. Among the studied genes, PAR-2 and HO-1 were significantly altered after the hunting period. To date, the relationship between exercise and increased HO-1 expression has been well-documented in different tissue and animal models (31–34). Such an increase is likely to restore HO-1 protein expression levels after 60 days of training (T1), when oxidative stress is high, as confirmed by the TAC data. As for PAR- 2, it is well-described in intestinal models of I/R injury that the receptor is strongly

activated by the tryptase released, for the most part by the mast cell infiltrate, with a consequent increase in paracellular permeability by means of the activation of myosin light chain kinase (MLCK) and myosin phosphatase (MP) (17, 90); once activated, the receptor is translocated to the lysosomes and degraded (23, 91). In different animal models regarding intestinal I/R injury, an increase in the PAR-2 transcript has been observed (21–23), consistent with the present data showing a slight but significant increase in PAR-2 mRNA levels at the end of the hunting period (T2). This similar trend in different models may be due to the fact that during exercise, the blood flow is diverted from the gut to the periphery, creating an I/R-like scenario (92) with the potential consequent activation of PAR-2. It has been reported that PAR-2 activation may directly affect cytoskeleton contraction by triggering the phosphorylation of MLCK with subsequent changes in TJ permeability, as demonstrated in *in vitro* epithelial models (19, 20). However, the unchanged expression level of OCLN suggests that the PAR-2 receptor activation in our model is insufficient to induce damage at the TJ level, and so we were unable to predict the impairment of barrier permeability.

The lack of the detection of cytokine transcripts and the absence of changes in CALP mRNA levels additionally reinforced the authors' assumptions, i.e., that physical exercise in the present model could be considered mild and did not result in a strong inflammatory GI response.

Nevertheless, metabolomics data indicate that some intestinal disorder occurred. A two-step approach regarding the metabolome of the feces, based on univariate/multivariate analyses, allowed hypothesizing the overall trends that the fecal molecule profiles underwent as a consequence of resting, training, and hunting. The samples collected at T2, T3, and T0 showed median scores along PC1 of −1.39, −0.12, and 3.69, respectively. From a metabolomic perspective, therefore, the recovery of baseline conditions seemed to be linearly related to time. The metabolomes of the dogs at rest before the training (T0) were markedly different from all the other time points. The greatest modifications from this long period of rest were associated with training, while the subsequent activities seemed to lead to a progressive return of the metabolome to the baseline characteristics. This confirmed a metabolic shift between rest and activity. Of the molecules leading to such a circular trend, some, as expected, pertained to the biochemical processes connected to energy (46). This was the case for malate, which is part of the TCA cycle. Interestingly, of the sugars, glucose showed no significant differences, while fucose and galacturonate did. Of the molecules that were, for the most part, modified in the present study, 1,3-dihydroxyacetone, formate, and uridine should be mentioned. In a previous experiment (93), these three molecules were found to be altered in mouse feces after the administration of probiotics, probably as a result of the modification of the intestinal microbiota. In particular, the increase in 1,3-dihydroxyacetone, an intermediate in fructose metabolism, was found to lead to an increase in intestinal permeability, which is a known consequence of prolonged strenuous exercise in both dogs (94) and humans (7).

Consistent with the abovementioned assumptions, the gut microbiota structure also underwent a rearrangement during training and tended to approach the initial configuration in the rest period following

the hunt. In line with the literature available regarding exercise and gut microbiota, this rearrangement was characterized by: (1) a tendency toward increased biodiversity (95); (2) decreased relative abundance of widely prevalent commensals (i.e., *Prevotella* and *Ruminococcaceae* members) (96–98); and (3) increased proportions of subdominant taxa, including *Streptococcus*, *Enterococcus*, and *Slackia* (96, 99). The majority of these changes were transient, which additionally reinforced the hypothesis of a reversible non-drastic alteration of the intestinal ecosystem. However, this was not true for *Streptococcus*, which, similarly to *Enterococcus*, includes species known to act as pathobionts, i.e., capable of pathogenic expansion under unfavorable conditions, compromising and eventually translocating across the epithelial barrier, with potentially severe implications for the host health (100). It is also worth noting that *Streptococcus* spp. are capable of proteolytically interacting with PARs (101) and have previously been positively correlated with uridine levels, probably by means of the activity of cytidine deaminase (102), which suggests a major role for this bacterial genus in exercise response. On the other hand, negative correlations have so far been found between uridine as well as DHA and *Bacteroides* (103), the relative abundance of which tended to be gradually reduced over the course of activity and no longer restored. Although transient and non-significant, the depletion of *Faecalibacterium*, a well-known butyrate producer with multiple health-promoting activities (104), constitutes another red flag for possible GI (and systemic) complications and should be monitored in cases of intense and prolonged physical activity.

CONCLUSION

The aim of the present explorative study was to evaluate the presence of a GI derangement in hunting dogs through a non- invasive sampling as a consequence of a period of intense exercise in comparison with samples collected at rest.

We evaluated a number of potential stress markers in canine fecal samples. In particular, FCMs, IgA levels, and the TAC were measured. Moreover, the expression of selected genes was investigated, and microbiota and metabolomics analyses were carried out. Exercise induced a variation in gene expression, a reduction in TAC, and a modulation of the microbiome and metabolome profiles. Despite the intense physical activity required for hunting wild boar, the animals did not seem to show signs of particularly high stress under conditions of programmed training; all the data were consistent with a limited degree of alteration of intestinal homeostasis. Despite the limited statistical power of the study related to the relatively low number of subjects enrolled, the present findings are encouraging for the development of a non-invasive monitoring method for detecting the effect of exercise in dogs using a multidisciplinary integrated approach.

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2 CLINICAL PRESENTATION

2.1 BREED, SEX, AGE

Chronic GI symptoms can occur for various causes in dogs of every age, breed, sex, but prevalence has been identified for CIEs.

The important role played by genetic in the pathogenesis of CIEs has been supported by studies that have identified breed disposition, however the triggering mutations are still in the research field [Kathrani et al., 2011; Simpson & Jergens, 2011; Peiravan et al., 2018;]. The main breed predisposition to different pathologies affecting the GI system found over the years are summarized in Table2.a.

A predisposition to develop enteropathies seems to exist in females but only in some races, particularly in Yorkshire Terriers [Bota et al., 2016] and Soft Coated Weaton Terrier with PLE [Littman et al., 2000], and in animals that have undergone gonadectomy [Sundburg et al., 2016]. However, in general, there is no evidence of a sex predisposition for the development of CIE in dogs [Kathrani et al., 2011].

CIEs can occur at any age, but the categories FRE and ARE appears to be more common in younger dogs than IRE [Allenspach et al., 2007; Allenspach et al., 2016; Dandrieux, 2016]. AFRs are more common in young dogs, even less than 1 year old, while ARE is usually more common in middle-aged dogs [Gaschen & Merchant, 2011; Dandrieux, 2016]. The form IRE, NRE, PLE are more frequent in older dogs, although in some predisposed breeds, for example Yorkshire Terriers, they can even occur at young age [Craven et al., 2004; Simmerson et al., 2014]. Therefore, it is more likely that young dogs will have a resolution of symptoms already with a dietary change while for older dogs it is more likely that antibiotic and/or immunosuppressive therapy is required [Allenpach et al., 2007; Dandrieux, 2016].

Breeds	Findings	References
German Shepherd Dogs	IgA deficiency, predisposition to develop ARE, alterations of TRL4 and TRL5	German et al., 2000; Allenspach et al., 2010; Kathrani et al., 2010; Peiravan et al., 2018.
Border Collies, Boxers, Golen Retrivers, Labrador Retrivers, Rottweilers, Weimaraners, West Highland White Terriers	Predisposition to develop CIEs	Craven et al., 2004; Kathrani et al, 2011; Allenspach et al., 2016.
Yorkshire Terriers, Rottweiler	Predisposition to develop PLE	Lecoindre et al., 2010; Dijkstra et al., 2010; Simmerson et al., 2014; Bota et al., 2016.
French Bulldogs, Boxers	Histiocytic Ulcerative Colitis (HUC) by *Escherichia coli*	Craven et al., 2011; Manchester et al., 2013.
Irish Setters	Gluten sensitivity enteropathy	Manners et al., 1998; Biagi et al., 2019
Soft Coated Weaton Terriers	Predisposition to develope PLE and Protein-Losing Nephropaty (PLN)	Littman et al., 2000; Allenspach et al., 2008.
Chinese Shar Peis, Giant Schnauzer Dogs, Border Collies, Australian Shepherd Dogs, Beagles	Cobalamin deficiency due to malabsorption	Battersby et al., 2005; Fyfe et al., 2014; Gold et al., 2015; Grützner et al., 2015; Hanisch et al., 2018.
Basenji, Shiba dogs	Lymphocytic Plasmatic Enteritis	MacLachlan et al., 1988; Ohno et al., 2006; Ohmi et al., 2011.
Norwegian Lundehunds	Lundehund Syndrome	Berghoff et al., 2007.

Table 2.a: Main breed predisposition to chronic GI disease in dogs.

IgA: Immunoglobulin A; TRL: Toll-like Receptor; CIE: Chronic Inflammatory Enteropathy; ARE: Antibiotic-Responsive Enteropathy; PLE: Protein-Losing Enteropathy; HUC: Histiocytic Ulcerative Colitis; PLN: Protein-Losing Nephropathy

2.2 CLINICAL SIGNS AND SCORES

The most frequent clinical signs in dogs with CIE are vomiting, diarrhea and weight loss. Less common symptoms are tenesmus, hematochezia and intestinal borborygmus. Other clinical signs are often associated with poor prognosis, such as hypoalbuminemia, thromboembolism, cavity effusion and/or peripheral edema, hypocalcemia [Allenspach et al., 2007; Simpson & Jergens, 2011; Schmitz et al., 2019]. There are clinical scores to target the severity of the symptoms of the patient which, associated to the state of nutrition assessed by the Body Condition Score (BCS) (Table 2.c) and the consistency of the stool evaluated by the Fecal Score index (FS) (Table 2.d), help to stage the case under examination. These scores are Canine Inflammatory Bowel Disease Activity Index (CIBDAI) and Canine Chronic Enteropathy Clinical Activity Index (CCECAI) (Tables 2.e) [Allenspach et al., 2007; Schmitz et al., 2019].

When investigating dog symptoms, it is important to distinguish between vomiting and regurgitation. Vomiting is defined as the emission of gastric content from the oral cavity as consequence of retching and usually nausea, while the expulsion of material from the oral cavity without retching is defined regurgitation. Regurgitation is typically associated with esophageal disease, but in some cases of CIE with dysmotility disorders there may be also regurgitation [Whitehead et al., 2016]. The presence, in the emitted material, of live or digested blood is usually associated with gastric disease such as erosions or ulcers. Yellow-colored bile vomit is the result of reflux of duodenal content in the gastric lumen, with consequent mucosal irritation. Can be a consequence of motility disorders resulting from inflammation of GI tract that predispose dogs to bilious vomiting syndrome (BVS), especially when they are fasted [Ferguson et al., 2016].

Diarrhea is defined as an increase in the frequency, fluidity, or volume of feces and it is one of the most frequent symptoms of CIE. Based on the frequency and some characteristic of the feces emitted, the origin of diarrhea can be localized distinguishing between small bowel diarrhea and large bowel diarrhea as summarized in Table 2.b [Marks, 2013]. In most cases, patients suffer from a mixed type of diarrhea, suggesting widespread bowel involvement. To objectively define the fluidity of the stool, and evaluate any improvements or worsening over time, a scoring system was created as reported in Table 2.d.

Sign	Small Bowel	Large Bowel
Frequency	Normal 2-3 times x day	>3 times x day
Vomiting	May be present	May be present
Urgency	Uncommon	Common
Volume	Increased	Decreased
Weight loss	Common	Uncommon
Dyschezia	Absent	Often present
Mucus	Rare	Common
Blood	Melena	Hematochezia
Steatorrhea	May be present	Absent
Tenesmus	Rare	Common

Table 2.b: Differentiation between small and large bowel diarrhea based on clinical signs and characteristics of feces. Modified from: Marks, 2013.

Weight loss is a consequence of malabsorption/maldigestion that occurs during CIE. It is often associated with diarrhea, but in rare cases it can be present in normal-formed stools if colon is not affected by inflammation (Table 2.e). Severe weight loss, ≥30%, is a negative prognostic factor (Table 2.b) [Ohno et al., 2006; Allenspach et al., 2007].

In curse of CIE often the appetite does not undergo alterations, but in some cases, animals affected, may present a decrease in appetite up to anorexia or, in other cases, polyphagia. Further alterations can include the ingestion of foreign material, called “pica”, that can lead in surgery in severe cases due to ingestion of foreign bodies [Yamada et al., 2019; Mills et al., 2020].

PLE dogs may have other clinical signs, less frequent and usually considered prognostic clinical factors, related to the state of protein loss. Ascites, fluid accumulation within peritoneal cavity, pleural effusion or peripheral edema are less frequent clinical signs found in course of hypoalbuminemia. Usually, it is pure or modified transudates but also chylous ascites has been reported [Lecoindre et al., 2010; Dossin & Lavouè, 2011; Simmerson et al., 2014]. Studies reported a hypercoagulable state in PLE dogs associated with reduced antithrombin III (AT III) plasma concentration, increased thrombin-antithrombin complexes or abnormal thromboelastogram, with consequent thromboembolic events [Goodwin et al., 2011; Jacinto et al., 2017; Sakamoto et al., 2020]. A further finding may be hypocalcemia, associated with lymphangiectasia, which, in the most serious cases, can lead to seizures [Whitehead et al., 2015; de Brito Galvão et al., 2017].

BODY CONDITION SCORE

5 and 9 point scale

5 Point	9 Point	Description
1/5	1/9	**Dogs:** Ribs, lumbar vertebrae, pelvic bones and all bony prominences evident from a distance. No discernible body fat. Obvious loss of muscle mass.  **Cats:** Ribs visible on short-haired cats; no palpable fat; severe abdominal tuck; lumbar vertebrae and wings of ilia obvious and easily palpable. 
1.5/5	2/9	**Dogs:** Ribs, lumbar vertebrae and pelvic bones easily visible. No palpable fat. Some evidence of other bony prominence. Minimal loss of muscle mass. **Cats:** Shared characteristics of BCS 1 and 3.
2/5	3/9	**Dogs:** Ribs easily palpated and may be visible with no palpable fat. Tops of lumbar vertebrae visible. Pelvic bones becoming prominent. Obvious waist.  **Cats:** Ribs easily palpable with minimal fat covering; lumbar vertebrae obvious; obvious waist behind ribs; minimal abdominal fat. 
2.5/5	4/9	**Dogs:** Ribs easily palpable, with minimal fat covering. Waist easily noted, viewed from above. Abdominal tuck evident. **Cats:** Shared characteristics of BCS 3 and 5.
3/5	5/9	**Dogs:** Ribs palpable without excess fat covering. Waist observed behind ribs when viewed from above. Abdomen tucked up when viewed.  **Cats:** Well proportioned; waist observed behind ribs; ribs palpable with slight fat covering; abdominal fat pad minimal. 

5 Point	9 Point	Description
3.5/5	6/9	**Dogs:** Ribs palpable with slight excess fat covering. Waist is discernible viewed from above but is not prominent. Abdominal tuck apparent. **Cats:** Shared characteristics of BCS 5 and 7.
4/5	7/9	**Dogs:** Ribs palpable with difficulty; heavy fat cover. Noticeable fat deposits over lumbar area and base of tail. Waist absent or barely visible. Abdominal tuck may be present.  **Cats:** Ribs not easily palpable with moderate fat covering; waist poorly distensible; obvious rounding of abdomen; moderate abdominal fat pad. 
4.5/5	8/9	**Dogs:** Ribs not palpable under very heavy fat cover, or palpable only with significant pressure. Heavy fat deposits over lumbar area and base of tail. Waist absent. No abdominal tuck. Obvious abdominal distension may be present. **Cats:** Shared characteristics of BCS 7 and 9.
5/5	9/9	**Dogs:** Massive fat deposits over thorax, spine and base of tail. Waist and abdominal tuck absent. Fat deposits on neck and limbs. Obvious abdominal distention.  **Cats:** Ribs not palpable under heavy fat cover; heavy fat deposits over lumbar area, face and limbs; distention of abdomen with no waist; extensive abdominal fat pad. 

Table 2.c: Body Condition System (BCS) for dogs and cats. Scores 2,5-3/5 and 4-5/9 are considered normal. Modified from: Baldwin et al., 2010

FECAL SCORE 7 point scale		
SCORE	SPECIMEN EXAMPLE	CHARACTERISTICS
1		· Very hard and dry · Often expelled as individual pellets · Requires much effort to expel from body · Leaves no residue on ground when picked up
2		· Firm, but not hard, pliable · Segmented in appearance · Little or no residue on ground when picked up
3		· Log shaped, moist surface · Little or no visible segmentation · Leaves residue on ground, but holds form when picked up
4		· Very moist and soggy · Log shaped · Leaves residue on ground and loses form when picked up
5		· Very moist but has a distinct shape · Present in piles rather than logs · Leaves residue on ground and loses form when picked up
6		· Has texture, but no defined shape · Present as piles or spots · Leaves residue on ground when picked up
7		· Watery · No texture · Present in flat puddles

Table 2.d: Fecal score (FS) for dogs. Scores 2 and 3 are considered normal. Modified from: Fecal Scoring Chart, Purina ProPlan Veterinary Diet

CIBDAI	CCECAI
Attitude/Activity ▪ 0 normal ▪ 1 slightly decreased ▪ 2 moderately decreased ▪ 3 severely decreased	Attitude/Activity ▪ 0 normal ▪ 1 slightly decreased ▪ 2 moderately decreased ▪ 3 severely decreased
Appetite ▪ 0 normal ▪ 1 slightly decreased ▪ 2 moderately decreased ▪ 3 severely decreased	Appetite ▪ 0 normal ▪ 1 slightly decreased ▪ 2 moderately decreased ▪ 3 severely decreased
Vomiting ▪ 0 normal ▪ 1 mild (1 x week) ▪ 2 moderate (2-3 x week) ▪ 3 severe (>3 x week)	Vomiting ▪ 0 normal ▪ 1 mild (1 x week) ▪ 2 moderate (2-3 x week) ▪ 3 severe (>3 x week)
Stool consistency ▪ 0 normal ▪ 1 slightly soft feces ▪ 2 very soft feces ▪ 3 watery diarrhea	Stool consistency ▪ 0 normal ▪ 1 slightly soft feces ▪ 2 very soft feces ▪ 3 watery diarrhea
Stool frequency ▪ 0 normal ▪ 1 slightly (2-3 x day)/fecal blood/mucus ▪ 2 moderately increased (4-5 day) ▪ 3 severely increased (>5 x day)	Stool frequency ▪ 0 normal ▪ 1 slightly (2-3 x day)/fecal blood/mucus ▪ 2 moderately increased (4-5 day) ▪ 3 severely increased (>5 x day)
Weight loss ▪ 0 none ▪ 1 mild (5%) ▪ 2 moderate (5-10%) ▪ 3 severe (<10%)	Weight loss ▪ 0 none ▪ 1 mild (5%) ▪ 2 moderate (5-10%) ▪ 3 severe (<10%)
Total: ▪ 0-3: clinically insignificant ▪ 4-5: mild ▪ 6-8: moderate ▪ ≥9: severe	Albumin levels ▪ 0 albumin >20g/L ▪ 1 albumin 15-19.9g/L ▪ 2 albumin 12-14.9g/L ▪ 3 albumin <12g/L
▪	Ascites and peripheral edema ▪ 0 none ▪ 1 mild ascites or peripheral edema ▪ 2 moderate amounts of ascites/peripheral edema ▪ 3 severe ascites/pleural effusion and peripheral edema
▪	Pruritus ▪ 0 no pruritus ▪ 1 occasional episodes of itching ▪ 2 regular episodes of itching, stops when asleep ▪ 3 dog regularly wakes up for itching
▪	Total: ▪ 0-4- Clinically insignificant ▪ 5-6 Mild ▪ 7-9 Moderate ▪ 10-12 Severe ▪ >12 Very severe

Table 2.e: CIBDAI and CCECAI score for dogs. Modified from: Allenspach et al., 2007.

CIBDAI: Canine Inflamamtory Bowel Disease Activity; CCECAI: Canine Chronic Enteropathy Clinical Activity Index

3 DIAGNOSTIC WORKUP

The diagnosis of CIE is based on the recognition of clinical signs, exclusion of other pathologies that determine a similar symptomatology and definition of the severity of clinical case under examination [Wennogle et al., 2017]. Therefore, collateral examinations and diagnostic imaging are necessary and, in case of a confirmed diagnosis of CIE, therapeutic trial is required to identify which phenotype the form of CIE belongs to. Usually the last step, before the immunosuppressant therapy, is the execution of an endoscopy of the digestive tract with acquisition of biopsies of each tract. However, in the most serious cases, once excluded other causes, the early stages of the trial, diet and antibiotic, are bypassed and endoscopy is proceeded directly, if the clinical conditions allow it [Dandrieux, 2016].

3.1 FECAL EXAMINATION

Coprological examination is performed first as GI parasites can cause chronic symptoms. Fecal flotation for the most common worms and protozoa (Giardia) should be performed on multiple stool samples, at least 3 collected in subsequent days, to increase the accuracy of the result [Dossin, 2011]. Parasites do not always give clinical symptoms and the coprological examination does not always identify parasites, therefore if there is a strong suspicious that there may be parasitosis, and especially if the dog is young, could be recommended to carry out an antiparasitic treatment [Dossin, 2011; Zanzani et al., 2014]. Performing antigenic and PCR tests for Giardia and Cryptosporidium can be useful in some cases, although Giardia often gives subclinical infestations in dogs [Sotiriadou et al., 2013; Tysnes et al., 2014], or, conversely, positive results of ELISA fecal tests may not be related to the disease [Olson et al., 2010].

Fecal culture can be useful only in some cases of acute diarrhea. Bacteria that are usually searched for as cause of diarrhea, in particular *Campylobacter* and *Salmonella*, can also be found in healthy dogs, thus making very difficult to interpret a fecal culture and reducing its usefulness for diagnostic purpose [Broussard, 2003; Werner et al., 2020].

3.2 BASIC LABORATORY WORKUP

Among the tests that are routinely performed there are blood count, biochemical profile, with hepatic and renal parameters and proteinemia, and urine analysis including proteinuria. In less severe cases, and in particular in dogs with FRE, there may be no alterations or in any case there may be slightly nonspecific alterations [Allenspach et al., 2007].

Alterations that can be found in course of CIE at the blood count are anemia, eosinophilia, lymphopenia, thrombocytosis. Although infrequent, anemia is usually mild and may be due to iron deficiency,

because of chronic GI blood loss, or to the chronic inflammatory state that occurs [Marchetti et al., 2010]. The finding of eosinophilia can be linked either to the presence of parasitosis or allergic forms or in cases of eosinophilic enteritis [Sattasathuchana & Steiner, 2014]. Lymphopenia is a frequent finding in dogs with PLE as a consequence of lymphangiectasia [Schmitz et al., 2019]. An additional finding in dogs with PLE is thrombocytosis, particularly in Yorkshire Terriers [Bota et al., 2016]. In some cases a mild neutrophilia with or without left shift can be found [Simpson & Jergens, 2011]. A recent study evaluated the Neutrophil-to-Lymphocyte Ratio (NLR), parameter well known in human medicine as a negative prognostic factor for some pathologies, highlighting an increased NRL in the most severe forms of CIE in dogs [Benvenuti et al., 2020c].

The first parameters to be evaluated in the biochemical examination are total proteins and albumin. Hypoalbuminemia is a characteristic finding of PLE with albumin value <2g/l. Hypoprotidemia, total protein value <5g/l, is usually due to reduced appetite, intestinal malabsorption, GI bleeding or exudation of protein. Mild hypoalbuminemia in other CIEs can also occur, and in both CIEs and PLE during treatment, if responsive, it increases [Allenspach et al., 2007; Gianella et al., 2017; Craven & Washabau, 2019]. To exclude a Protein-Losing Nephropathy as cause of hypoalbuminemia, a urinalysis including urinary protein:creatinine ratio (UPC) should be performed. For the malabsorption and lymphangiectasia that dogs with CIE can suffer, some common alterations can be hypocholesterolemia, hypotriglyceridemia, reduction of Antithrombin III with possible thromboembolism [Goodwin et al., 2011; Schmitz et al., 2019]. Liver enzymes (ALT, AST, SAP) may be slightly increased during CIEs, due to non-specific reactive hepatitis linked to impaired intestinal absorption, but liver function, investigated by serum bile acids (BA) and ammonia, is usually preserved [Hall, 2013]. Iron deficiency is usually linked to GI blood loss, such as in course of erosions or ulcers, but also malabsorption and inflammatory state can also contribute to this alteration [Marchetti et al., 2010]. Total and ionized calcium are often reduced, especially during PLE. Total calcium is affected by the albumin value, therefore ionized calcium is more reliable. This reduction may be associated with hypomagnesemia, especially in PLE dogs [Kimmel et al., 2000; Simmerson et al., 2014; Whitehead et al., 2015]. Closely related to calcium metabolism is vitamin D, which will be discussed in the chapter 3.3. C Reactive Protein (CRP) during CIE is usually not much increased, except for septic complications linked to the intestinal pathology [Jergens, 2004]. Alterations in electrolytes, sodium, potassium, chlorine, are usually found only in cases of severe symptoms with severe GI losses and may be associated with increased renal parameters (pre-renal azotemia) for dehydration [Hall, 2013]. In this case, urinalysis is necessary to exclude concomitant pathologies. Higher blood urea concentration seems to be associated with a negative outcome in dogs with PLE [Kathrani et al., 2019].

Further parameters that may be required to investigate the case in question are Trypsin-like Immunoreactivity (TLI), Canine Pancreatic Lipase Immunoreactivity (cPLI), Folate and Cobalamin. TLI and cPLI are useful to exclude exocrine pancreas insufficiency (EPI) and pancreatitis, with a negative prognostic role of the cPLI in dog with CIE in which it is increased [Kathrani et al., 2009; Dossin, 2011]. Folate (vitamin B9) is a water-soluble vitamin which serum concentration can be increased in course of CIE due to excessive bacteria

production, for example during dysbiosis, or reduced if there is malabsorption (Figure 3.a) [German et al., 2003; Dossin, 2011]. According to a recent abstract, folate can be altered for several GI pathologies, not only CIEs, and have not been correlated with the severity of the pathology and the outcome [Petrelli & Schmitz, 2020]. Cobalamin (vitamin B12) is a water-soluble vitamin absorbed in the ileum by the intrinsic factor, a specific carrier, produced by the pancreas and the stomach (Figure 3.b). Decreased serum cobalamin is a frequent finding in dogs with EPI or CIE and in certain breeds (see chapter 2.1, Table 2.a). In CIE hypocobalaminemia is a negative prognostic factor, so oral or parenteral supplementation is needed [Allenspach et al., 2007; Grützner et al., 2012; Toresson et al., 2016].

Finally, given that 1 out of 25 dogs with chronic GI symptoms is affected by hypoadrenocorticism, the basal cortisol value should always be measured. ACTH stimulation test is indicated when basal cortisol value is ≤2µg/dL to exclude Addison disease [Wakayama et al., 2017; Hauck et al., 2020].

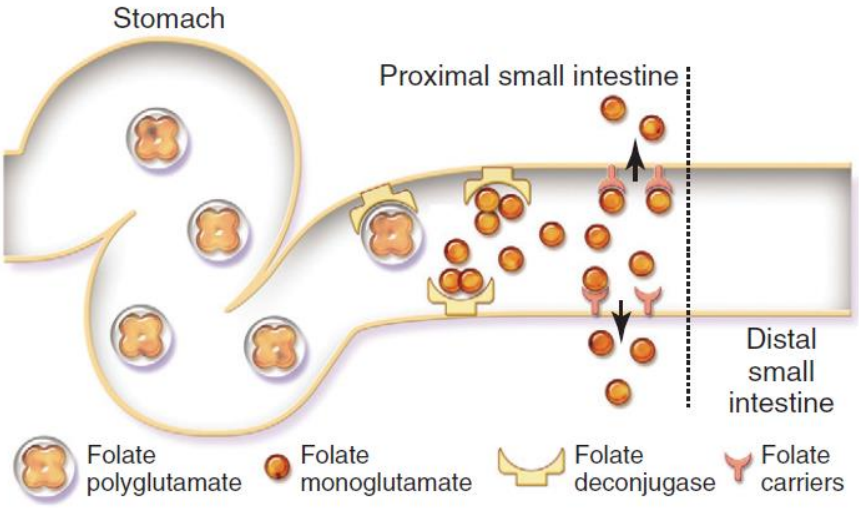


Figure 3.a: Absorption of folate. Folates are normally present in diet, once deconjugated by specific enzyme in the jejunum they are capture by specific carriers and absorbed. Modified from: Suchodolski et al., 2012.

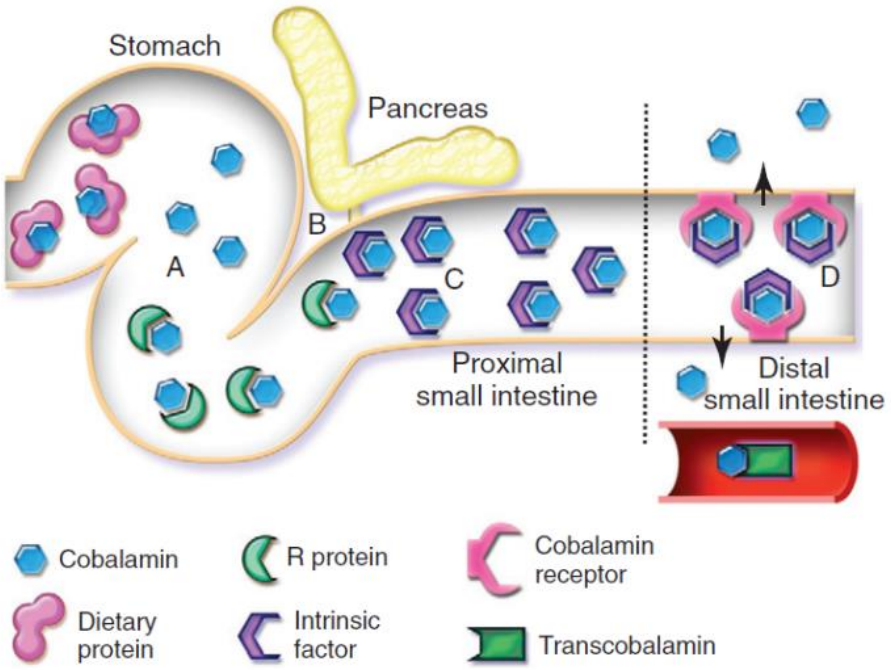


Figure 3.b: Absorption of dietary cobalamin. Cobalamin in diet is bound to dietary protein, after gastric digestion is realized and immediately bounded to R-protein, secreted in saliva and gastric juice. Pancreatic enzymes release cobalamin from R-protein in the duodenum. Intrinsic factor, produced from pancreas and stomach, binds cobalamin to the ileum where this complex is absorbed by specific receptors. Modified from: Suchodolski et al., 2012

3.3 ADDITIONAL EXAMS

There are additional tests that can be performed in dogs with CIE, but they are usually not performed routinely due to the high cost, difficulty in preserving the biological sample or small number of laboratories that perform the examination. The following are the main additional exams with some hints of new markers being validated.

Serum hypovitaminosis D is considered a negative prognostic factor in dogs with CIE, in particularly PLE [Titmarsh et al., 2015b; Allenspach et al., 2017]. The pathogenesis of this deficiency is not fully understood, although a multifactorial etiology has been recently proposed that includes reduced intake due to decreased appetite, fat malabsorption, intestinal loss, and reduction due to pro-inflammatory state [Titmarsh et al., 2015a; Wennogle et al., 2019]. The role of calcitriol supplementation in these subjects however requires further studies [Allenspach et al., 2017].

Fluorescence in Situ Hybridization (FISH) highlights the presence of bacterial DNA or RNA identifying genus and specimen of the bacteria involved in a biopsy fragment. *Escherichia coli* associated with HUC was identified by FISH in colon biopsies [Simpson et al., 2006]. Therefore, it is a recommended test in breeds predisposed for this form of CIE [Craven et al., 2011; Manchester et al., 2013].

Calprotectin and S100A12 are two calcium-binding proteins that have been identified as fecal biochemical markers of intestinal inflammation during CIE. Increased concentrations of these proteins is correlated with severity of clinical signs, response to treatment, endoscopic lesions, and intestinal histologic alterations [Heilmann et al., 2016b; Heilmann et al., 2018].

More and more importance is given to the composition of intestinal microbiota with the development of methods for the research of dysbiosis. Some of the most used methods are FISH, quantitative real-time PCR (qPCR), next-generation sequencing (e.g. 454-pyrosequncing, Illumina), metagenomics (shotgun sequencing of genomic DNA) [Suchodolski, 2016]. A dysbiosis index (DI) has been developed based on a numerical value obtained from a qPCR panel of 8 bacterial strains: *Faecalibacterium, Turicibacter, E.Coli, Streptococcus, Blautia, Fusobacterium e C. hiranonis*. This index could provide information on the progress of the microbiota over time, especially in correlation with therapy [Suchodolski, 2016].

Alpha1-Proteinase Inhibitor (α1-PI) is an endogenous serum anti-proteinase that can be loss into the intestinal lumen as consequence of PLE or GI blood loss. It can be found in feces as it resists to bacterial degradation, so it can be a useful test to diagnose PLE in dogs [Heilmann et al., 2016a].

In human IBD, alterations in serum amino acids (AA) that perform protective functions in the intestinal mucosa have been demonstrated, in particular arginine, glutamine, glycine, cysteine, N-acetylcysteine, and proline. Diagnostic and therapeutic investigations are therefore focusing on these markers. In recent years, the role of these AA in CIE has also been investigated in veterinary medicine, finding alterations in serum AA values in dogs with PLE and IRE compared to healthy controls [Kathrani et al., 2018; Benvenuti et al., 2020a].

However, further studies are necessary to define the role of these AAs as a diagnostic marker and above all to evaluate their use as therapeutic integrations.

Various clinical conditions may involve changes in serum homocysteine (HCY), although the specific mechanisms are not yet fully understood. Serum HCY in dogs with IRE was recently evaluated, showing an increase. The results of the study showed a negative correlation between serum HCY concentration and cobalamin regardless of folate concentrations. However, no association was found between serum HCY levels and clinical, endoscopic or histological scores, also suggesting further studies to investigate the role of HCY in dogs with CIE [Benvenuti et al., 2020b].

3.4 DIAGNOSTIC IMAGING

Radiographic examination of the abdomen can provide useful information, such as the presence of GI foreign bodies in the case of pica. Ultrasound examination of the abdomen is an indispensable step, both to exclude non-gastrointestinal pathologies and to characterize the enteropathic patient. Ultrasound alterations that are frequently encountered during CIEs are hyperechogenicity of the mucosa, lymphangiectasia (Figure 3.c), abdominal lymphadenopathy, increased thickness of the intestinal wall, loss of distinction of stratigraphy, alterations of peristalsis. Finally, with ultrasound it is possible to highlight the presence of free peritoneal fluid, usually associated with intestinal inflammation, or ascites, usually resulting from hypoalbuminemia [Sutherland-smith et al., 2007; Larson & Biller, 2009]. Ultrasound is essential to exclude tumors of the GI tract or particular forms of CIE such as lipogranulomatous lymphangitis [Watson et al., 2014].

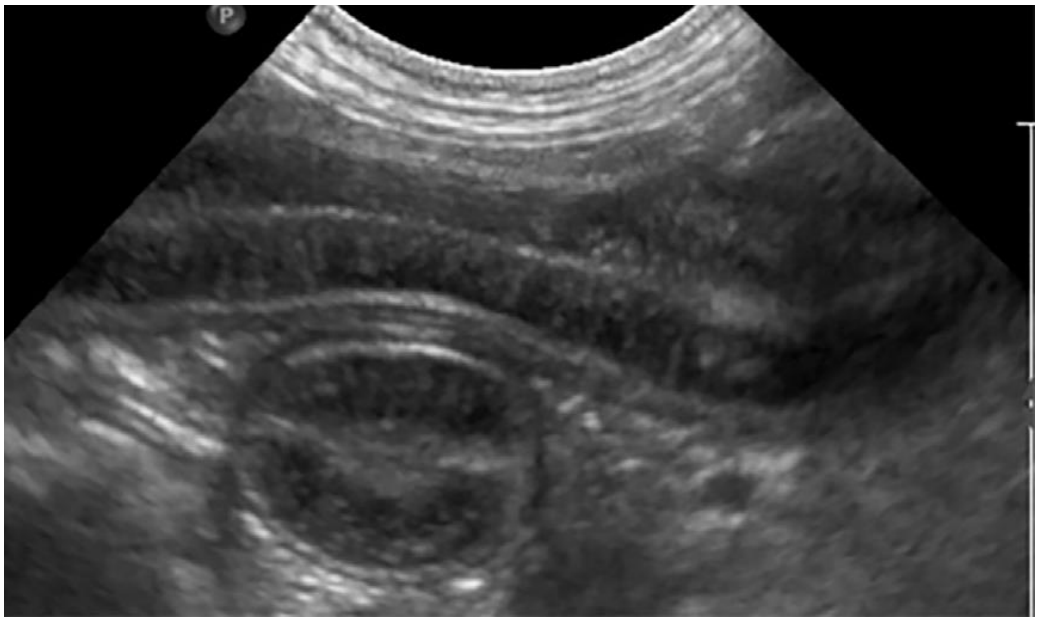


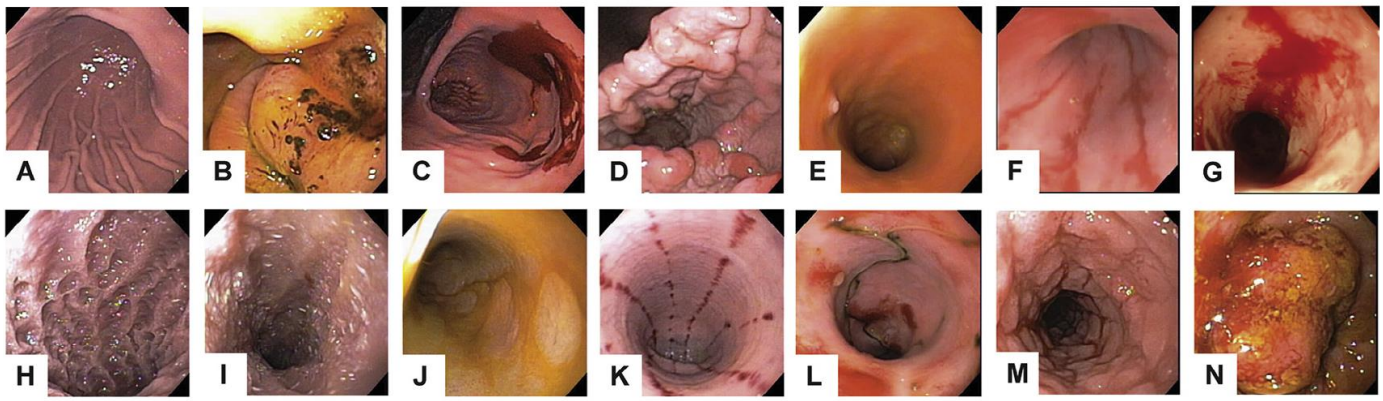
Figure 3.c: Ultrasonographic image of hyperechoic mucosal striations. Several radial hyperechoic mucosal striations indicative of villous lymphangiectasia are visible, indicative of lacteal dilation. From: Procoli, 2020.

3.5 ENDOSCOPY

Endoscopic examinations in dogs with CIEs is usually performed after excluding the FRE and ARE forms and therefore, in case of clinical failure to diet change and antibiotic therapy, before proceeding with immunosuppressive therapy (IRE subtypes). Recently, however, the use of antibiotic in course of CIE has been discouraged, thus making the endoscopic examination the next step to dietary trial, to try to counter the problem of the antibiotic resistance [Cerquetella et al., 2020]. GI tract endoscopy should be performed quickly in cases of severe PLE and in breeds at risk of HUC with symptoms referable to the large intestine, such as French Bulldogs and Boxers, always with the exclusion of non-GI causes [Washabau et al., 2010]. Even in the case of severe symptoms such as hematemesis, melaena and severe ferrous anemia or regurgitation with suspect of esophagitis, endoscopy becomes a priority [Chamness, 2013]. Endoscopy is a minimally invasive and rapid examination however it requires general anesthesia, therefore the need to perform it in patients with critical clinical conditions must be evaluated [Washabau et al., 2010]. Endoscopy of the GI tract, for diagnostic purpose and for the collection of biopsy samples, must be performed not only on the upper digestive tract (gastro-duodenal endoscopy - GD), but also including the lower tract, (gastro-duodenal-ileum-colonoscopy (GDIC), with particular attention to the jejunal/ileal tract. Performing GDIC endoscopy involves a more complete exploration of the gastrointestinal tract, even if the maximum part of the jejunum remains

unexplored in any case. However, it involves a longer anesthesia and a more complex preparation of the patient with prolonged fasting and possible administration of laxatives.

The endoscopic evaluation serves both to confirm the severity and localization of the problem (e.g. site of erosions, lymphangiectasia, presence of masses) and to acquire biopsies of each tract [García-Sancho et al., 2011; Adamovich-Rippe et al., 2017]. Biopsy samples should be 8-10 for each tract examined and histological analysis serves to certify the severity and type of inflammatory infiltrate as well as to exclude neoplasms (e.g. intestinal lymphoma) [Gieger, 2011; Jergens et al., 2016]. To make the visual assessment of the endoscopist as objective as possible, scores have been created to help in the classification of the severity of the GI alterations found in endoscopy [Washabau et al., 2010; Slovak et al., 2015]. In the Endoscopic Activity Score (EAS) for each characteristic of the GI mucosal a score is given ranging from 0 to 2, where 0 is absence of the lesion and 2 is a severe lesion (Figure 3.c).



Figue 3.c: Representative still images in the development phase of the endoscopic study. (A) normal stomach; (B) gastric erosions; (C) gastric friability; (D) gastric granularity; (E) normal duodenum; (F) duodenal erosions; (G) duodenal friability; (H) duodenal granularity; (I) duodenal lymphatic dilatation; (J) normal colon; (K) colonic erosions;(L) colonic friability; (M) colonic granularity; (N) colonic mass. From Slovak et al., 2015.

3.6 HISTOLOGY

The purpose of the histopathologic evaluation of biopsy specimen is to distinguish normal to pathologic tissue, to characterize the nature and severity of the alterations, and to provide a diagnosis on the type of inflammation. It is important that biopsy samples are in sufficient number and adequate size and storage, and that there is a correct interaction between clinician and pathologist, favoring the obtaining of useful data for the clinician, both for the prognosis and for the choice of and adequate therapy [Willard & Mansell, 2011; Washabau et al., 2010; Allenspach et al., 2019]. The infiltrate most found in CIEs is lymphoplasmacytic type, other forms that can be found are neutrophilic, usually associated with infectious causes, and eosinophilic, which usually has a worse prognosis [Sattasathuchana et al., 2014; Wennogle et al., 2017].

It can be hard to distinguish severe forms of lymphoplasmacytic enteritis from lymphoma, especially with endoscopic biopsy samples. In some doubtful cases it is therefore recommended to associate the histological evaluation with the execution of further methods, to distinguish between inflammation and neoplasm, such as immunohistochemistry (IHC), for B-cell and T-cell markers, or detection of antigen receptor gene rearrangement by polymerase chain reaction (PARR) [Gieger, 2011; Ohmura et al., 2017].

For the histological evaluation, as for clinical and endoscopy scores, there are scores to objectify as much as possible the detected alterations. These scores are based on an increasing number to attribute based on the severity of infiltration and lesions found, with a recent addition of ileus assessment to the previous WSAVA guidelines [Washabau et al., 2010; Allenspach et al., 2019].

INCIDENCE OF BACTEREMIA CONSEQUENT TO DIFFERENT ENDOSCOPIC PROCEDURES IN DOGS: A PRELIMINAR STUDY

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SIMPLE SUMMARY: Antimicrobial resistance is a threat that poses a great risk to public health. It has been predicted that, by 2050, there will have been 10 million deaths worldwide due to drug-resistant infections. There is a crucial need in Veterinary Medicine to reduce the use of antimicrobials to slow down the process and incidence of antimicrobial resistance as a One Health concern. The aim of this study was to evaluate the appearance of bacteremia following endoscopic procedures in dogs brought to the Veterinary Teaching Hospital (VTH) of the Department of Veterinary Medical Science of the University of Bologna. The results obtained from hemocultures before and after the endoscopic procedures demonstrated a low incidence of bacteremia after endoscopy. This could be seen as an attempt to reduce the use of antimicrobials to avoid the spread of antimicrobial resistance.

ABSTRACT: Endoscopic procedures are widely used in veterinary medicine, and their role in producing transient bacteremia is debatable. The growing issue of antibiotic resistance requires the correct use of antibiotics, avoiding their administration when not strictly necessary. Studies highlighting post-endoscopy bacteremia in veterinary medicine are extremely rare and often involve very few animals. This study describes the results from 74 owned dogs, brought to the Veterinary Teaching Hospital of the Department of Veterinary Medical Science of the University of Bologna, for the purpose of undergoing an endoscopic procedure. Two blood samples were taken from each dog, one before and one after the procedure, in order to assess the incidence of bacteremia linked to endoscopic procedures. Eight dogs were tested positive at the second blood culture with an Incidence Risk (IR) of 10.8%. No statistical differences were found by comparing positive and negative blood cultures with respect to sex, age, weight and anesthesia duration. In addition, no difference was found between airway and digestive tract procedures. The present findings showed that the probability of developing bacteremia after an endoscopic procedure was quite low, and additional studies confirming this are certainly recommended as well as the evaluation of categories of patients potentially considered at risk.

Key words: endoscopy; bacteremia; incidence risk; risk ratio; dogs; veterinary teaching hospital

INTRODUCTION

Endoscopic procedures in veterinary medicine are performed for both diagnostic (i.e., gastrointestinal and respiratory disease) and therapeutic (such as the removal of foreign bodies) purposes [1–6].

Local or remote tissue infections are complications that may follow an endoscopy. These could be the result of mucosal trauma with consequent alteration of the physiological barriers, and bacterial translocation of endogenous microbial flora into the bloodstream [7,8].

The growing issue of antimicrobial resistance strengthens the necessity of correctly using these drugs, avoiding their administration when not strictly needed [9,10]. The incidence of bacteremia linked to endoscopic procedures has been assessed in human medicine by several studies which showed a low percentage, up to 8%, of short-lasting transient bacteremia in these patients [7,11–13]. Considering these results, it is possible to draw up guidelines, in human medicine, for antibiotic prophylaxis during endoscopies that permit administration of antibiotics only in patients considered at risk (patients with high-risk cardiac conditions or gastrointestinal tract infections) or undergoing procedures considered at high risk (esophageal bougienage or PEG/PEJ tube placement) [8].

Studies regarding post-endoscopy bacteremia in veterinary medicine are extremely rare [14,15]. Therefore, there are no specific guidelines regarding preventive antibiotic prophylaxis for dogs and cats undergoing endoscopic procedures, leaving the decision up to the clinician.

The aim of this preliminary prospective study was to evaluate the incidence of bacteremia related to endoscopies in dogs, to assess whether or not antibiotic prophylaxis is required for such procedures in veterinary medicine.

MATERIALS AND METHODS

Owned dogs undergoing endoscopic procedures, brought to the Veterinary Teaching Hospital (VTH) of the University of Bologna from November 2015 to June 2017, were included in the study. The endoscopic procedures considered were either for diagnostic or therapeutic purposes: rhinoscopy, bronchoscopy, gastroscopy and gastro-duodeno-colonoscopy.

Dogs over six months of age and weighing more than 3 kg (kg) were included. All dogs underwent routine pre-anesthetic hematology tests, carried out using an automated hematology system (ADVIA 2120, Siemens Healthcare Diagnostics, Tarrytown, NY, USA). Dogs having anemia, immunodeficiency or sepsis based on the reference intervals provided by Moritz et al. [16] as well as those having concurrent pathologies or symptoms except those related to the endoscopy were excluded. Dogs who had undergone immunosuppressive and/or antibiotic treatments within the previous three weeks were also excluded, except for those in therapy with tylosin.

To assess the incidence of bacteremia, as the result of mucosal trauma, linked to endoscopic procedures, two blood samples were taken for blood culture, the first immediately before the beginning of

the procedure and the second twenty minutes after the end of the procedure. Both blood samples were taken with the venous catheter used for the anesthesia in order to eliminate additional painful and stressful manual manipulation.

To allow adequate functioning of the blood culture system chosen for the study (Signal™ Blood Culture System (Oxoid) Waltham, MA, USA), the minimum quantity of blood to be taken was 3 mL and the maximum was 8 mL. Blood volumes were established based on the welfare of the dogs and the blood culture system limit threshold.

The circulating blood volume in a dog is approximately 85 mL per kilogram (mL/kg) of body weight. The maximum amount of blood that can be collected from a dog for a transfusion donation, without causing hemodynamic alterations, is 19 mL/kg corresponding to approximately 17–22% of the total blood volume [17]. Therefore, in order not to cause any suffering, dogs weighing less than 3 kg were excluded from the study and, for those between 3 kg and 10.5 kg, the minimum amount of blood required for the blood culture system was collected at each time point. The dogs were divided into three groups based on body weight: (1) 3–10.5 kg, (2) 10.6–25 kg, (3) >25 kg. For each group, a different amount of blood was drawn: 3 mL in Group 1; 5 mL in Group 2; 8 mL in Group 3.

Each step was performed under sterile conditions by a trained operator, and the blood samples were obtained by means of a venous catheter allocated for the general anesthesia. The anesthetic protocol for each dog was chosen based on the temperament of the animal itself and the type of procedure required.

To position the intravenous catheter, the hair above the cephalic vein was removed. Subsequently, the skin was disinfected with three alternating passages of 4% chlorhexidine and denatured ethyl alcohol 70% (each left on for 30 s), following the main indications for skin antisepsis [18,19]. Once the area was ready, the intravenous catheter was positioned with the operator wearing sterile gloves. Following

the manufacturer's instructions, the blood samples were collected, and were immediately aseptically injected into the Signal™ Blood Culture System (Oxoid) and incubated at 36 ± 1 ◦C.

The blood cultures were routinely inspected twice a day for a total incubation period of at least 7 days. A positive result was indicated when the blood/broth mixture was forced into the signal device by the pressure created by growing organisms. The positive blood cultures were subcultured by streaking 20 µL of the blood/broth mixture onto one plate of Blood Agar (BBL) and were incubated in aerobiosis. Furthermore, two plates of Columbia Blood Agar (BD) were incubated in anerobiosis and in microerophilia, respectively. The incubation temperature was 36 ± 1 ◦C for all plates. To isolate any metabolically weak bacteria which were not able to activate the signal device, blind subcultures were obtained from the negative blood cultures at the end of the 7th day by applying the same conditions used for the positive ones. All the bacterial strains isolated were identified first phenotypically using Analytic Profile Index (API) test systems (bioMérieux) and were then confirmed genotypically by partial sequencing of the 16S rRNA gene [20].

The trial was authorized by the Animal Welfare Committee of the University of Bologna (Protocol N. 1173 of 11/06/2020).

Statistical Analysis

The data collected at the time of hospitalization were entered into a, MS Excel spreadsheet, merged with the microbiological results, then imported into Stata 15 (StataCorp LLC, College Station, Texas, TX, USA) for the analyses. Sex, age by month, weight, anesthesia duration and endoscopic procedures were the variables grouped. The endoscopies were first analyzed as a dichotomous variable, being divided into the airway and digestive tract procedures. Thus, 5 groups were formed and assessed: gastroscopy, gastro-duodeno-colonoscopy, rhinoscopy, bronchoscopy and rhinoscopy + bronchoscopy. Incidence risk was interpreted as the probability of a dog developing bacteremia owing to the endoscopic procedure. The non-normally distributed continuous data were summarized using medians and interquartile ranges (IQRs), while the normally distributed data were summarized using mean ± SD. When reasonable, the continuous variables were categorized. Pearson's $\chi 2$ test was used to compare the hemoculture results and the categorical variables. The Fisher's exact *P*-value was considered as more than 20% of the cells had expected frequencies <5 [21]. The Risk Ratio (RR) and relative 95% confidence interval (CI) were also calculated to evaluate whether tylosin was a protective factor for developing bacteremia. The Kruskal–Wallis non-parametric test was carried out when non-normal distributed continuous variables were involved. The results were considered to be significant when $p \leq 0.05$.

RESULTS

Overall, seventy-four dogs were included in the study. A total of 148 hemocultures were carried out. There were 31 males (42%), 17 females (23%), 7 castrated males (9%) and 19 neutered females (26%) included, the median age was 46 (IQR 21–86) months (range 5–179). The weight ranged from 3.7 to 56 kg (mean 22.2, SD + 10.1). Twelve dogs belonged to Group 1 (16%), 33 to Group 2 (45%) and 29 to Group 3 (39%). Ten out of the 74 dogs were under tylosin treatment (13.5%). Blood samples were obtained before and after performing 74 endoscopic procedures which included 20 gastroscopies (27%), 17 gastrointestinal endoscopies (23%), 18 rhinoscopies (24.3%), 11 bronchoscopies (14.9%) and 8 rhinoscopies + bronchoscopies (10.8%). The median duration of the anesthesia procedures was 45 min (min 15–max 240, IQR 30–80). The distribution of the different variables is summarized in Figure 1.

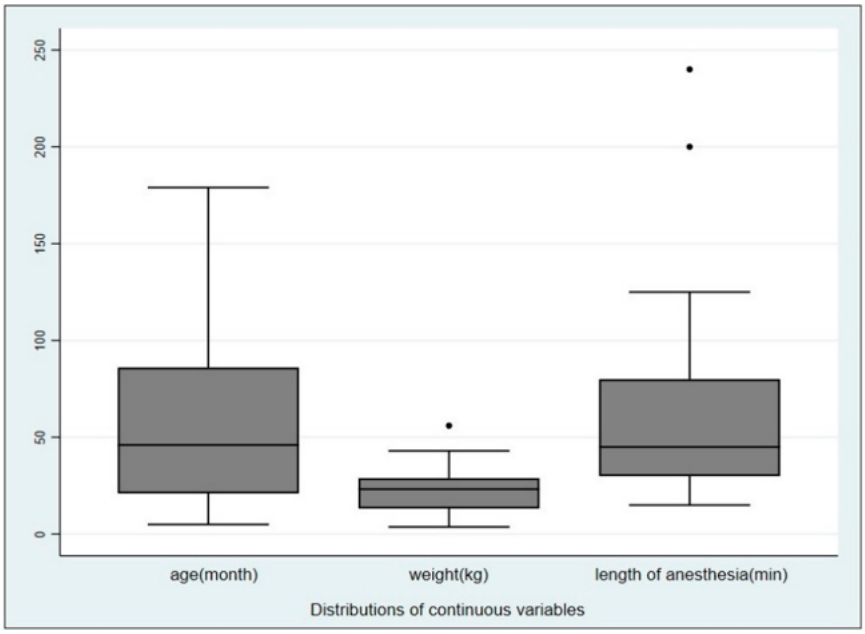

Figure 1: Boxplot of age, weight and length of the anesthesia procedures in the population sampled, displaying the distribution of data.

Only dogs with a negative pre-procedure blood culture and a positive post-procedure blood culture were considered as positive for the statistical analysis.

Patients in which pre-procedure results were positive and the post-procedure results were negative or positive were considered to be negative for the statistical analysis, since the results were not attributable to the endoscopic procedure (see Table 1).

Animals	Pre-Procedure Result	Post-Procedure Result	Procedure Attributable Result
n = 53	−	−	−
n = 8	+	+	−
n = 5	+	−	−
n = 8	−	+	+

Table 1: Interpretation of the hemoculture results attributable to the procedures.

+ tested positive to the blood cultures; – tested negative to the blood cultures.

A total of 66 dogs were negative. Of those, twenty-seven were males (41%), 17 females (26%), 16 neutered females (24%) and 6 castrated males (9%). The median age was 44 (IQR 21–90) months (range 5–179). The mean weight was 22.6 ± 10.1 kg (3.7–56). Finally, the median length of anesthesia was 47.5 (IQR 30–80) mins (range 15–240).

Eight dogs were positive at the second blood culture (10.8%). Four males, one castrated male and three neutered females were bacteremia-positive. The mean weight was 12.2 ± 10.6 kg (range 8.8–40.7), the

median anesthesia duration was 34.5 (IQR 22.5–80), ranging from 20 to 100 min. The median age was 60.5 (IQR 33.5–82.5) months, ranging from 21 to 149 months. The bacteria isolated were: *Pseudomonas aeruginosa* (3 dogs), *Propionibacterium acnes* (2 dogs), *Staphylococcus warneri* (1 dog), *Staphylococcus pseudointermedius* (1 dog), *Aeromonas hydrophila* (1 dog).

When comparing the positive and the negative blood cultures regarding sex, age and weight, no statistical differences were found. Endoscopic procedures were analyzed both as dichotomous and as categorical and none of the variables showed any statistical difference. All variables, values and results are summarized in Table 2.

Categorical Variables	Categories	# of Patients	%	Pearson χ2	Fischer's Exact *p*-Value	Degrees of Freedom
Sex	Male	38	51.3	0.446	0.387	1
	Female	36	48.7			
Age	≤12 months	9	12.2	4.2351	0.273 *	3
	>12 ≤57	34	45.9			
	>57 ≤84	12	16.2			
	>84	19	25.7			
Weight	≥3 kg ≤10.5 kg,	12	16.0	0.946	0.614 *	2
	≥10.6 kg <25 kg,	33	45.0			
	≥25 kg	29	39.0			
Endoscopy procedures (dichotomous)	airways	37	50.0	2.242	0.261	1
	digestive tract	37	50.0			
Endoscopy procedures (categorical)	gastroscopy	20	27.0	3.724	0.284 *	4
	gastro-duodeno-colonoscopy	17	23.0			
	rhinoscopy	18	24.3			
	bronchoscopy	11	14.9			
	rhino+broncho	8	10.8			

Table 2: Pearson χ2 comparison between the categorical variables and the hemoculture results.

* Overall *p*-value.

Ten dogs were under treatment with tylosin at the time of the procedure; relative risk was calculated as RR = 0.914, resulting in a weak protective factor but still not significant (95% CI: 0.13–6.66).

Finally, based on the non-parametric Kruskal–Wallis test ($h = 0.933$, $p = 0.333$), the anesthesia duration did not statistically affect the probability of developing bacteremia after the procedure.

DISCUSSION

The aim of this prospective study was to evaluate the incidence risk of bacteremia following endoscopic procedures of the respiratory and gastrointestinal tracts.

The first sampling was useful in demonstrating that the dog was not previously bacteremic before endoscopy. In fact, transient bacteremia can arise following ordinary daily activities without causing any symptoms [22].

Eight subjects showed negative blood cultures before the procedure and were positive at the end of the endoscopy. These cases were considered as bacteremic consequent to the execution of the endoscopic

procedure. The bacteria isolated were found to be normal inhabitants of cutaneous and upper airway microbial flora. Thus, it was reasonable to hypothesize that these positive results could be associated with mucosal trauma and subsequent translocation of native microorganisms. There were no significant differences concerning the onset of bacteremia regarding sex, age, body weight, procedure duration or type of endoscopy. Furthermore, eight patients were positive for the same bacterial strain in both blood cultures, and five patients were positive at the first blood culture and negative at the second. These patients were considered negative as the positivity was not considered to be related to the endoscopic procedures. In these dogs, the isolated microorganisms were found to be bacteria belonging to normal canine microflora or contaminants, such as *Serratia marcescens* [23]. Even using aseptic manual manipulation, a certain percentage of blood culture contamination is also described in

human medicine [24–26].

Dogs who had taken antibiotics or immunosuppressants in the 20 days prior to the endoscopy were excluded from the study, with the only exception being tylosin therapy. Until recently, tylosin was part of the therapeutic and diagnostic trial of canine chronic enteropathies [27]. After the tylosin trial, non-responsive patients underwent gastro-duodeno-colonscopy without suspending the tylosin-treatment. In order to not exclude this wide group of patients, we felt to consider them for analysis. However, the results reported that tylosin had a weak protective factor (RR = 0.914) for developing bacteremia after the procedure, but it was still not significant.

Antimicrobial prophylactic treatment, in human medicine, is only recommended for patients considered at risk of developing complications following transitory bacteremia, such as infectious endocarditis. The high-risk category includes patients suffering from severe cardiac conditions, those who are immunocompromised or those undergoing high risk-procedures, such as esophageal dilation and sclerotherapy [28,29]. In human medicine, performing gastrointestinal or respiratory tract endoscopies did not statistically affect the incidence risk of developing bacteremia [11–13].

The authors' findings showed that the probability of developing bacteremia consequent to endoscopic procedures of the respiratory and gastrointestinal tracts in the dog population in this study was quite low, with an incidence risk of 10.8%. This is in agreement with the American Society for Gastrointestinal Endoscopy which reports gastroscopy in humans as a low risk-procedure with an incidence of bacteremia ranging from 0% to 8%, with or without carrying out a biopsy [28]. To the authors' knowledge, only two similar studies have been carried out on dogs, reporting an incidence of 17.9% and 3%, respectively [14,15]. Li et al. [14] used dogs as an animal model to assess bacteremia after Endoscopic Ultrasonography-guided Fine Needle Aspiration (EUS FNA) of the pancreas in humans. Authors reported an incidence of bacteremia around 18%. The higher value compared to our one was perhaps due to the more invasive procedure performed. Although the dogs utilized were experimental-dogs, a high frequency of contamination was observed as a consequence of the high amount of bacteria present on the dog's hair which can translocate through the intravenous catheter.

The second study [15] aimed to evaluate whether the use of proton pump inhibitors in dogs favored the onset of bacteremia following a gastroscopy. Differences in the incidence of bacteremia could have been due to the low number of samples included in the other study and to the inclusion of only gastroduodenoscopies as compared to the variable procedures in the present study. Moreover, Jones et al. [14] used research dogs having almost the same body weight, and the same amount of blood was drawn for each culture. Finally, the samples were taken without the aid of an intravenous catheter.

After performing the endoscopy, the choice of whether to start antibiotic therapy was evaluated by the veterinarian, based on the animal's endoscopic diagnosis and clinical situation, and not on the result of blood culture. None of the patients developed symptoms attributable to infections or septicemia after the procedures.

The study had some limitations. First, an additional blood culture was not carried out hours after the end of the endoscopy to evaluate the short duration of the bacteremia caused by the procedures. In the majority of cases, bacteremia caused by endoscopic procedures is of short duration and is most frequently observed within the first 30 min after the end of the procedure [7,22]. Since the animals in this study were not experimental animals, after the complete awakening from the anesthesia, the intravenous catheter was removed, and the patients were discharged from the hospital. Therefore, a third blood culture, could not be carried out using the venous catheter; moreover, another blood sample would have increased the amount of blood taken from each animal, impacting the dog's hemodynamics. To eliminate additional painful and stressful manual manipulation, it was decided to use the intravenous catheter in order to minimize the impact on the animals, even if this represented another limitation. In fact, transient bacteremia can occur due to various causes, including the insertion of an intravenous catheter. Despite maintaining sterile conditions, the intravenous catheter could also be a bacterial adhesion site [30,31]. The choice of reducing the amount of blood for each blood culture, related to the well-being of the dogs, was an additional limitation as it may have affected the result of the blood cultures. The quantity of blood and the timing and methods of incubation are, in fact, important for obtaining a reliable result when carrying out a blood culture [32]. However, to minimize the occurrence of false-negative results, we used a strong conservative approach re-culturing negative blood cultures under the same conditions as the positive ones.

CONCLUSIONS

In conclusion, to the best of the authors' knowledge, there are no guidelines regarding preventive antibiotic therapy following these endoscopic procedures in dogs. These preliminary results tended to exclude the preventive use of antibiotics following endoscopic procedures, unless related to the disease diagnosed. However, additional studies confirming the low incidence and transient nature of the bacteremia are certainly recommended, as is including and evaluating the categories of dogs potentially considered at risk.

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WATER IMMERSION VS. AIR INSUFFLATION IN CANINE DUODENAL ENDOSCOPY: IS THE FUTURE UNDERWATER?

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ABSTRACT

Endoscopy represents a commonly employed technique for canine enteropathies. Different trials in human intestinal endoscopy have suggested that the introduction of water for luminal distension, in place of air, improves the visualization of the mucosal texture and decreases pain.

The aim of the study was to compare water immersion (WI) vs. air insufflation (AI) during duodenoscopy in anesthetized dogs in terms of mucosal visualization and nociception.

Twenty-five dogs undergoing duodenoscopy were included. The same image of the descending duodenum was recorded applying WI and AI. Each pair of images was analyzed using morphological skeletonization, an image entropy evaluation, and a subjective blind evaluation by three experienced endoscopists. To evaluate differences in nociception related to the procedure applied, heart rate and arterial blood pressure were measured before, during and after WI/AI. To compare the two methods, a t-test for paired data was applied for the image analysis, Fleiss' Kappa evaluation for the subjective evaluation and a Friedman test for anesthetic parameters.

No differences were found between WI and AI using morphological skeletonization and entropy. The subjective evaluation identified the WI images as qualitatively better than the AI images, indicating substantial agreement between the operators. No differences in nociception were found.

The results of the study pointed out the absence of changes in pain response between WI and AI, likely due to the sufficient control of nociception by the anesthesia. Based on subjective evaluation, but not confirmed by the image analysis, WI provided better image quality than AI.

Key words: dog, duodenoscopy, water immersion, air insufflation, image analysis, nociception

INTRODUCTION

Endoscopy of the gastrointestinal tract is a routine examination in human and veterinary medicine for the diagnosis of acute and chronic enteropathies (Jergens et al. 2016).

In order to allow the visualization of the intestinal mucosal surface, it is necessary to dilate the lumen, by using air, or better carbon dioxide, insufflation as a dilation media (Xu et al. 2016). However, as demonstrated in human medicine, insufflation of gas during colonoscopy can increase the angulation and bending of the gut, making the procedure technically more difficult and more painful for the patient (Shah et al. 2002, Xu et al. 2016). For this reason, colonoscopy is often performed under sedation. Approximately twenty years ago in order to try to reduce the side effects of the procedure by allowing the examination in non-sedated sub- jects, Baumann introduced the water infusion-technique into human colonoscopy as an alternative to the use of air (Baumann 1999, Leung et al. 2007, 2009, Terruzzi et al. 2012). At present, in human medicine, two different methods involving the use of water are in use: water immersion (WI) and water exchange (Leung 2013). The WI technique employs water infusion for luminal distension, with limited use of air insufflation (Lee et al. 2012, Falt et al. 2013). The water infused in the intestinal lumen is generally aspirated during the retraction of the endoscope. The water exchange technique, by means of the infusion and the almost-simultaneous suction of water, involves a continuous recirculation of the liquid in the colon, thus maintaining a layer of clear water and allowing the progression of the instrument along the cecum without employing air. This technique minimizes the distension of the organ and maximizes the cleaning of the lumen during insertion (Cadoni and Isahaq 2018). Moreover, in addition to the subjective pain reduction, the water--infusion technique is able to determine an improvement in image quality (with respect to the employment of gas) which can be quantified by measuring the increase in diagnostic capacity, namely the ability to identify a mucosal neoplasia (the so-called adenoma detection rate) (Radaelli et al. 2010, Lee et al. 2014, Wang et al. 2015). Unlike the endoscopic procedures applied in human medicine, enteric endoscopy in veterinary medicine cannot be performed without the use of general anesthesia. Although the patient is not conscious, assessment of nociception during the procedure could be verified by detection of heart rate and arterial blood pressure modification. Therefore, the aim of this study was to carry out a blind study, comparing two endoscopic techniques, WI and Air insufflation (AI) during duodenoscopy in dogs, in terms of image quality assessment and evaluation of the signs of nociception by means of the detection of cardiocirculatory alterations (heart rate and arterial blood pressure).

MATERIALS AND METHODS

Inclusion and exclusion criteria

Dogs of different breeds, gender and ages requiring diagnostic duodenoscopy were included in the study. Animals were included if their body size allowed the passage of an endoscope having a diameter of 9.8

mm through the pylorus when performing a duodenoscopy. The recruitment of dogs for the study was voluntary and at no cost to the owners. Written informed consent before enrollment in the study was obtained by the owners.

Anesthetic protocol

The standard anesthetic protocol consisted of intra- muscular premedication with dexmedetomidine (Dextro- quillan®, Fatro S.p.A. Italy) (0.001 - 0.005 mg/kg) and butorphanol (Nargesic®, ACME S.r.l. Italy) (0.2 mg/Kg), followed by intravenous induction with propofol (Proposure®, Boehringer Ingelheim Animal Health Italia S.p.A. Italy) titrated to effect (1-4 mg/Kg). During the procedure, the patient was kept under anesthesia with isofluorane (Isoflo®, Esteve S.p.A. Italy) in oxygen.

Endoscopy

The endoscopic procedure consisted of a duodenoscopic examination carried out for diagnostic purposes. The animals were positioned in left lateral recumbency in order to allow easier trans-pyloric passage of the endoscope.

Endoscopic examination of duodenum was per- formed before the full examination of the stomach, to avoid paradoxic motion of the instrument and difficulty in crossing the pyloric sphincter, due to the stomach's distension.

The pattern of the mucosal surface, the shag carpet appearance created by the villi, the major (and occasionally the minor) duodenal papilla and the Peyer's patches were examined.

All the procedures were performed by the same operator (MP), employing the same endoscope (Pentax EG-2931 K, diameter: 9.8 mm).

Water immersion and air insufflation

The duodenal dilation was obtained with the use of both water (WI) and air (AI) in every dog, starting randomly with either the first or the second method. For WI, an amount of warm to the touch water (from 40 to 100 ml until the duodenal lumen was completely filled, depending on the size of the dog) was instilled in the descending duodenum with a syringe through the biopsy channel. If the procedure started with AI, WI was always preceded by air aspiration and, conversely, by water aspiration if the procedure started with WI. All the procedures were recorded using a soft- ware package (Pinnacle Studio 22 Plus, Corel Corp., Ottawa ON, Canada). When the lumen was completely dilated with water and the duodenal lumen became transparent, an image of the duodenum was captured and saved by means of dedicated computer software (Adobe Premiere Elements, Adobe Inc, San Jose, CA, US) (Fig. 1A). The water was then removed from the aspiration channel, the lumen dilated with air, and an endoscopic image of the same bowel portion was acquired (Fig. 1B). If the procedure started randomly with water, the steps were reversed.

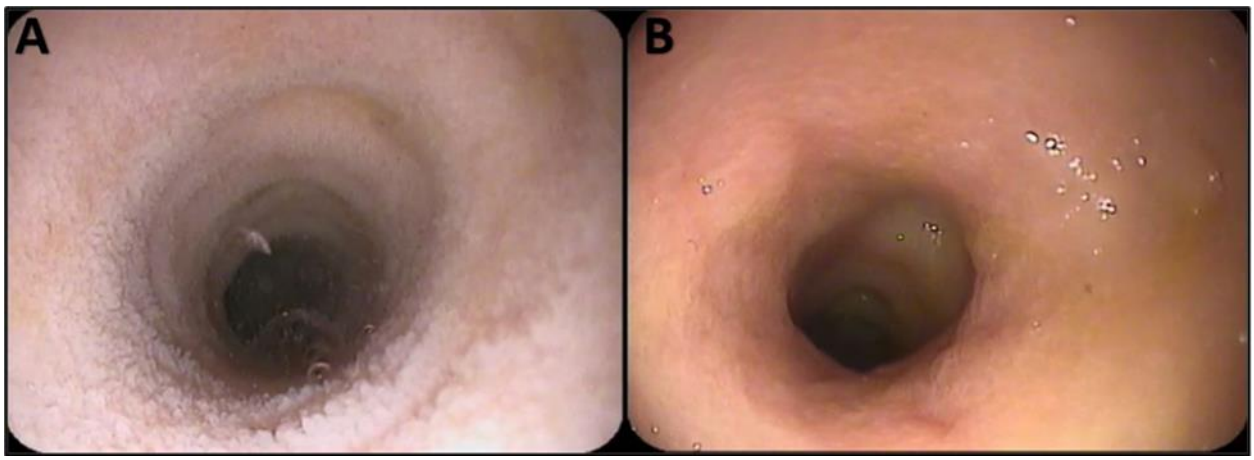

Figure 1. Example of the same images of the canine descending duodenum recorded with the two endoscopic methods described in the study (A: water immersion, B: air insufflation).

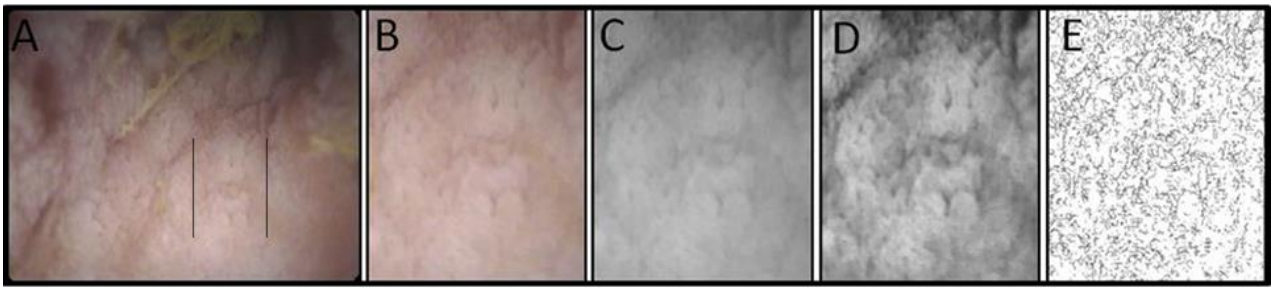

Figure 2. Example of the application of the texture analysis method (morphological skeletonization). The method employed used the ImageJ software using the Fiji plugin. The same image of the descending duodenum was recorded with AI and WI. From the original image (A), a homogenous and not blurred rectangular portion of the image was selected (B) and the area of the rectangle was calculated in pixels. The image was then converted to a gray scale with the function "RGB (Red, Green, Blue) to luminance" (C) and then contrast enhanced with the "CLAHE" function (D). The algorithm called "Find edges" was subsequently applied to highlight the sharp inten- sity changes of the image; the binary option was then used to obtain a binary image. Finally, the Skeletonize option (E) was applied, and the length of the image obtained was measured in pixels. The ratio between the rectangle and the skeletonized area was considered; of the two images (obtained with WI or AI); the image having a lower ratio was considered to be of the best quality.

Image analysis

All pairs of images acquired from each dog, record- ed under WI and AI, were analyzed both by applying two different computer analysis procedures (morpho- logical skeletonization and image entropy evaluation), and by a subjective blind evaluation. The first method, derived from a human medicine study in which the comparison, carried out on images of the duodenum of healthy patients vs. patients affected by celiac disease, was used to evaluate the degree of villous atrophy (Ciaccio et al. 2011). In brief, the images were analyzed by employing ImageJ software with the Fiji plugin (National Institutes of Health, Bethesda, Mary- land, US) (Figs. 2A-E). A homogenous and unblurred rectangular portion of each image was selected and its area was calculated in pixels. The RGB (Red, Green, Blue) image was then converted to a gray scale, and the contrast was enhanced. A high-pass "edge finding" filtering algorithm was subsequently applied in order to

highlight the sharp intensity changes of the image; a binary image was then obtained. Finally, a skeletonization procedure was applied and the lengths of the lines were measured in pixels. The ratio between the rectangle and the skeletonized area was calculated and compared between the two images (obtained with WI or AI); the image having a lower ratio was qualitatively higher in definition. The second method employed (Figs 3A-C) evaluated the entropy, defined as the amount of information given by an image. This method used ImageJ software with the GLCM (Gray-Level Co-occurrence Matrix) plugin for texture analysis (National Institutes of Health, Bethesda, Maryland, US). All the RGB images were converted to a gray scale, and a homogenous and unblurred rectangular portion was selected. The application of the GLCM algorithm allowed obtaining a numeric value, quantifying the entropy. Between the two images (obtained with WI or AI), the image characterized by lower entropy was considered to be of higher quality. Lastly, a subjective blind evaluation was carried out. Three endoscopists, each with a minimum of 5 years expertise in the field, participated in the study. All the endoscopists involved were external to the project. Each pair of images (WI vs. AI) underwent a blind evaluation carried out by the three experts (A; B; C) who independently selected the best image by analyzing three parameters: 1) mucosal texture, considered as villi definition; 2) sharpness of the eventual lymphangiectasia and 3) eventual granulation, defined as the presence of longitudinal grooves in the mucosa (Figs 4A-C). When comparing the two images, the operators had to give a score of 0 (worst image) or 1 (best image) for each parameter. After adding up the scores for each image, the image receiving the highest score was considered to be the best one. Therefore, for each pair of images, three distinct results were indicated, corresponding to the choice of each of the three experts; only at the end of the evaluation were the 75 (25 X 3) results thus obtained decoded and attributed to each of the two groups (WI and AI). No single evaluator was aware of the method applied in the image recording nor of the endoscopic diagnosis. It should be clarified that an expert endoscopist, based on his/her own experience, could differentiate between the two methods, but the evaluation was made considering the quality of each individual parameter in the images compared to try to transform the subjective judgement into an objective evaluation.

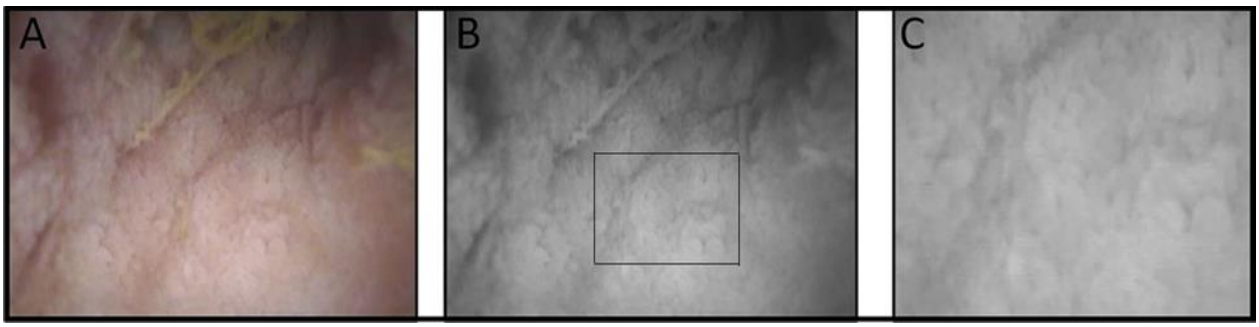

Figure 3. Example of the application of the texture analysis method (entropy). The method employed used ImageJ software with the GLCM (Gray-Level Co-occurrence Matrix) plugin for texture analysis. The same image of the canine descending duodenum was recorded using AI and WI (A). All the images were converted to a gray scale (function "RGB [Red, Green, Blue] to luminance") (B), and a homogenous and not blurred rectangular portion was selected (C). The application of the GLCM algorithm allowed obtaining a numerical value which quantified the entropy. Of the two images (obtained with WI or AI), the image characterized by lower entropy was considered to be of the best quality.

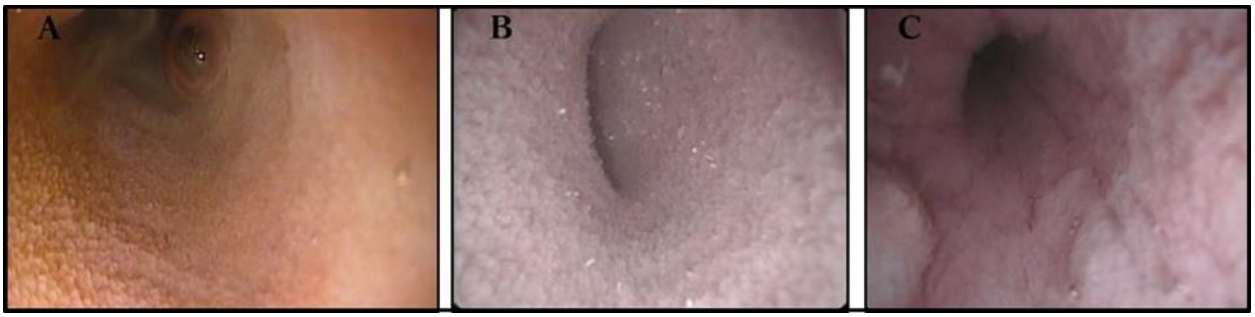

Figure 4. Example of the parameters considered for subjective evaluation; the first image (A) shows a portion of the canine duodenal lumen in which the villi profile can easily be defined on the mucosal surface, corresponding to a high definition of the mucosal texture; in the second image (B), there are several white spots distributed in the mucosa which correspond to severe cystic lacteal dilation. The third image (C) explains the granulation, defined as the presence of longitudinal grooves in the mucosa. In this image, they are evident in the right-ventral portion of the picture.

Cardiocirculatory parameters

For the purpose of the nociception evaluation and with the aim of achieving a stable anesthetic plane before each examination, the anesthesia and the endoscopic procedure were divided into four phases. Baseline (phase 1) began soon after the induction of the general anesthesia and ended with the beginning of the water/air phases. The water/air or air/water phases (phase II and III) consisted of the application of air or water, as dilation media, applied in a random order. Outcome (phase IV) was considered to be the end of the evaluation, beginning with water/air reabsorption. Each step was recorded for not less than one minute. During each phase, the heart rate was monitored using a Datex-Ohmeda S5 monitor (GE Healthcare Italia, Milano, Italy) and the blood pressure was measured from the brachial artery using a Pettrust sphygmomanometer (BioCare Corp., Taiwan, China). At baseline, the isoflurane vaporizer was adjusted in order to obtain a stable plane of anesthesia characterized by relaxed jaw tone, an absent palpebral reflex and a ventral position of the eyeball. The vaporizer setting was maintained constant throughout the four phases

of evaluation. In case of an insufficient anesthetic plane, a bolus of propofol was administered IV and its eventual administration was recorded. At the end of the outcome phase, the vaporizer was adjusted by the anesthetist in charge based on the patient's requirement in order to complete the endoscopic examination.

Statistical analysis

The statistical analyses were carried out using soft- ware MedCalc® 16.8.4 (MedCalc Software bvba, Ostend Belgium). Randomization of the procedure (WI vs AI) was carried out by applying a Fisher-Yates algorithm. Assessment of the data for normality was carried out applying the D'Agostino and Pearson Omnibus normality test. For the image analysis, a t-test for paired data was applied for morphological skeletonization and image entropy evaluation, while a Fleiss' Kappa evaluation was applied for the subjective evaluation. For the anesthesiological parameters, the median measured values during each of the four phases were compared using the Friedman test. Significance was set at $p<0.05$ for all the analyses.

Institutional animal care or other approval declaration

The study was approved by the University Scientific Ethics Committee for Experimentation on Animals (Approval No. 1087/2019).

RESULTS

A total of 25 dogs were enrolled in the study, 5 mixed breed and 20 pure breeds (3 Boxers, 2 German Shepherd dogs, 2 Miniature Dachshunds, 2 Miniature Poodles, 2 Pugs, 1 Bernese Mountain Dog, 1 Bull Mastiff, 1 Golden Retriever, 1 Irish Setter, 1 Maltese, 1 Miniature Pinscher, 1 Standard Schnauzer, 1 Samoyed and 1 Yorkshire Terrier). Thirteen were male [9 intact males (36%) and 4 neutered males (16%)], and 12 were female [3 intact females (12%) and 9 spayed females (36%)]. The age of the dogs ranged from 8 months to 13.5 years (mean 5.11 years) and body weight ranged from 3 to 42 Kg (mean weight: 19.4 Kg). The definitive diagnosis was lymphocytic-plasmacytic enteritis in 23 dogs, a gastric adenocarcinoma in 1 dog while no alterations on endoscopy and histologic examination were identified in one case. The time required to complete the WI procedure ranged from 60 to 318 seconds (mean 102±62.7 seconds). Air Insufflation is commonly performed during duodenoscopy; there- fore, it was not considered as an additional procedure in terms of time. Even considering the additional time, the total time of the anesthesia was influenced very little. No side effects related to the duodenal water instillation were evidenced during the trial and up to one hour after the examination.

Image analysis

All 25 pairs of images obtained during the duodenal dilation with water and air were compared. No significant difference was observed between the pairs of images with regards to morphological skeletonization (WI 0.12±0.03; AI 0.13±0.05; p=0.46), and image entropy evaluation (WI 5.65±0.41; AI

5.62±0.60; p=0.85). The subjective blind evaluation of each pair of images carried out by three experienced endoscopists (25 X 3 = 75 pairs of images), evaluating mucosal texture, and sharpness of the eventual lymphangiectasia and granulation, showed a significant predilection for the WI technique (71/75) as compared to the AI technique (4/75).

The Fleiss' K evaluation had a K value of 0.74 (range: 0.61 - 0.80), showing substantial agreement between the results provided by the endoscopy experts, supporting the WI method.

Cardiocirculatory parameters

The cardiocirculatory parameters analyzed referred to 24 dogs since one dog was excluded due to the lack of data at the fourth step (outcome). None of the dogs required an additional bolus of propofol throughout the four phases of the examination period. The mean fraction of expired isoflurane was 1.5 +/- 0.4 %. Heart rate and blood pressure values (systolic, diastolic, and mean) of the samples are reported in Table 1. With regard to the heart rate and blood pressure values, no significant differences were observed among the four different steps of the procedure (baseline, water, air, outcome).

PARAMETER	BASELINE			WATER			AIR			OUTCOME			*p*
	Median	Min	Max	Median	Min	Max	Median	Min	Max	Median	Min	Max	
HR (bpm)	90	49	164	79	47	180	82	42	174	82	49	155	0.829
SAP (mmHg)	106	80	157	110	75	189	105	71	141	109	78	135	0.511
DAP (mmHg)	65	49	130	64	45	137	61	44	113	63	44	108	0.299
MAP (mmHg)	79	60	141	78	57	153	77	56	156	80	55	120	0.358

Table 1. Cardiocirculatory parameters divided into four phases. There were no significant differences among the four phases in any of the parameters.

HR: heart rate; SAP: systolic arterial pressure; DAP: diastolic arterial pressure; MAP: mean arterial pressure; Min: Minimum; Max: Maximum

DISCUSSION

The study, which aimed at applying human endos- copy procedures to dogs, differed from studies on humans, primarily due to the part of the intestinal tract involved: the colon in humans vs. the duodenum in dogs. The reason for the different sites depends on the particular diagnostic needs characterizing the different species. In human medicine, endoscopy of the colon is a procedure frequently applied to the diagnosis of polyposis or colon cancer while, in veterinary medicine, endoscopic examination of the small bowel (duodenum and ileum) is more frequently requested for diagnosing chronic enteropathy (Jergens and Simpson 2012, Lee et al. 2014). When analyzing the results of the present study, it can first be noted that computer analysis of the endoscopic images obtained using water did not show any difference from those using air as a spacer. In the medical field, the use of texture analysis is a valid method of overcoming the subjectivity

provided by the operator's judgment (Holli et al. 2010). In particular, the choice of using image analysis (morphological skeletonization and image entropy evaluation) derives from studies carried out in human medicine, even if they involved other intestinal areas than the one analyzed in this study (Holli et al. 2010, Ciaccio et al. 2011, Cannellas et al. 2018). However, it is necessary to emphasize that no previous studies in veterinary medicine have compared these methods of image processing to collate two different procedures of enteric endoscopy. Moreover, unlike what had been reported in a previous study carried out in human medicine (Ciaccio et al. 2011), in the present study, the comparison was not carried out in order to differentiate between a pathological versus a healthy condition, but to compare two different endoscopic techniques applied to the same intestinal area in the same subject. Therefore, it is plausible that the differences between the two images were not as evident as they would be when comparing the enteric surfaces of healthy and sick patients.

In addition, the lack of statistical differences between the WI and the AI images, applying the skeleton measurement, could be linked to the quality of the images themselves which were derived from the freeze frames acquired from digital videos and, therefore, were often not perfectly in focus. On the other hand, the application of image entropy evaluation as an index of comparison between two images has already been applied in human medicine in order to differentiate between benign and malignant intestinal neoplasms (Cannellas et al. 2018). However, in the present study, the use of this analysis technique as a comparison approach did not provide any significant difference in relation to the media used to dilate the bowel, water vs. air. As previously discussed regarding morphological skeletonization, the reason for this result could be attributable to the quality of the images. The images obtained during an endoscopic examination are not well defined when compared to those obtained by other imaging procedures, such as computed tomography, which have already been successfully evaluated by entropy (Cannellas et al. 2018).

With regard to the images evaluated subjectively, those obtained by dilation of the lumen with water, when compared to those obtained using air, showed improved visualization of the duodenal surface with a higher degree of definition of the lymphangiectasia and of the granulation, if present.

The preference of the WI method, with respect to the AI method, was in agreement with numerous studies carried out in human medicine, which have shown how the use of the water technique could determine a greater cleaning of the intestinal lumen, leading to better visualization of the mucosal surface and greater distension of the villi (Fuccio et al. 2018), with an increase in polyp and adenoma detection (Cadoni et al. 2017, Cadoni and Leung 2017). In the present study applied to the duodenum, the cleaning of the mucosa was not as important as it is in the colon since the first intestinal tract is always cleaner than the large bowel. However, the better visualization under WI could depend on the fact that the villi did not collapse which is a common feature observed during AI endoscopy. An additional aim of the study was to evaluate nociception in relation to the application of water or air to dilate the intestinal lumen during endoscopy. In human medicine, unlike what happens in veterinary medicine, endoscopy is a procedure which can be performed without sedation, or

under a low degree of sedation, allowing patients to be conscious and reporting their own feelings of pain and discomfort (Bushnell et al. 2013, Al-Zubaidi et al. 2016). Therefore, based on the results obtained directly from non-anesthesized human patients, water-assisted endoscopy minimizes patient discomfort and reduces the need for sedation (Leung et al. 2007, 2009, Terruzzi et al. 2012, Xu et al. 2016). In canine patients, endoscopic evaluation is carried out only in anesthetized patients (McFadzean et al. 2017), and this obviously prevents the use of pain scales for objective pain evaluation which are applicable only in non-anesthesized animals (Reid et al. 2007). Even if, to the Authors' knowledge, nociception has never been evaluated in anesthetized dogs undergoing endoscopic examination, changes in heart rate and blood pressure have already been used for the evaluation of nociception in dogs during surgical procedures (Höglund et al. 2011, Caniglia et al. 2012). In the present study, neither the heart rate nor the blood pressure changed significantly with air insufflation vs water immersion. Considering this, in the population in the present study, the water technique did not have any advantage over the air-assisted technique in terms of nociception. However, it should be considered that all the dogs were premedicated with dexmedetomidine and butorphanol which produce analgesia and deep sedation in dogs (Nishimura et al. 2018). Therefore, it is believed that these drugs, along with propofol and isoflurane, provided a stable anesthetic plane and prevented changes in the physiologic parameters in spite of the stretching of the walls of the gastrointestinal tract which is considered to be the most painful stimulation induced by endoscopic examination. In fact, dexmedetomidine has already been described to provide hemodynamic stability, effective sedation and comfort for human patient undergoing colonscopy (Dere et al. 2010); therefore, it cannot be excluded that it also has the same effect on dogs. In addition, when heart rate and blood pressure were evaluated in human patients during colonscopy, various authors observed that propofol sedation pre- vented significant changes in these parameters which were, instead, observed in non-anesthesized patients (Gasparović et al. 2003).

It should be emphasized that this study has some limitations, the first being related to the quality of the images collected which could have influenced the results of the image analysis, and the second to the short time between the experimental phases. The latter may have limited the response of the animal to the mechanical insult of air and water. For ethical reasons, since the animals in the study were patients and not experimental animals, it was impossible to have a longer time bet- ween the phases because it would have extended the anesthesia for longer than what was required for the classic procedure.

The results of this study indicated that the introduction of water instead of air during duodenoscopy in dogs provided an increase in the quality of the endoscopic images, documented by subjective evaluation, although not by the texture analyses.

In conclusion, WI was a cheap, non-time consuming, easy to apply, alternative technique to AI endoscopy. Therefore, although no clinical post-WI evidence was found and no observable side effects were recorded during and one hour after the trial, additional studies are needed to investigate any possible general influence of the WI method.

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4 TREATMENT AND PROGNOSIS

The treatment of CIEs involves a therapeutic trial which, based on the clinical response of the dog, allows to classify the type of CIE from which it is affected. The steps include a dietary change, to be continued for a minimum period of about 3 weeks, the introduction of an antibiotic, usually tylosin but recently this step is replaced using probiotics to reduce antibiotic resistance, the execution of an endoscopy of the digestive tract with biopsy samples and the introduction of an immunosuppressant. The timing between each step of the trial can be reduced, or in some cases even bypassed, in the most severe cases where immunosuppressive therapy needs to be initiated. [Dandrieux, 2016; Cerquetella et al., 2020]. Below are briefly reported therapies known for dog CIEs with the most recent scientific updates and the main prognostic factors related to this group of pathologies.

4.1 DIET

A favorable clinical response to dietary change brings the subject in question into the FRE group. These patients are usually young and the symptoms are mainly referable to the large intestine [Bresciani et al., 2018]. Patients with FRE are not necessarily allergic or intolerant to food, even if we often talk about AFR, but the mechanism underlying the response to food change is not yet fully understood, although it seems that intestinal microbiota plays a role since different dietary compositions can modify it [Gaschen & Merchant, 2011; Craig, 2019; Mori et al., 2019].

The change of ingredients must be as radical as possible, precisely to eliminate any allergic component as the cause of gastrointestinal symptoms. In addition to selecting a protein different from the one previously taken or even better hydrolyzed, the diet should be formulated with high digestibility, a fat restricted content (preferably <15%), and a reduced number of ingredients in the formulation, in some cases a grain-free or vegetable proteins formulated diet can be recommended [Allenspach et al., 2007; Simpson & Jergens, 2011; Bresciani et al., 2018]. A composition with further restriction in the percentage of fat is what dogs with PLE need. PLE is a form of CIE in which there is dilatation of intestinal lymphatic that result in extravasation of protein-rich lymph and in decreased absorption due to the damage at the intestinal mucosa [Peterson & Willard, 2003; Dandrieux, 2016]. It has been demonstrated that in these subjects a low-fat diet or ultra-low-fat diet is part of the therapy, probably for a decreased lymphatic pressure [Okanishi et al., 2014; Nagata et al., 2020].

Commercial diets may contain traces of other foods or the composition of additives could be the trigger for gastrointestinal symptoms [Ricci et al., 2013]. A further option is therefore a homemade elimination diet, with the selection of a single protein and carbohydrate source. The advantages of the home diet are the palatability and the certainty of not having contamination, but it is essential that it is correctly balanced in

order not to worsen the deficiency states that patients with CIE may have [Dandrieux, 2016]. The BARF (bone and raw foods) diet, very popular among owners in recent years, is certainly not suitable for patients with CIE. The formulation is often not balanced and presents risks related to the ingestion of bones as well as representing a danger for some infectious diseases, including zoonotic ones, related to the ingestion of raw food [Schlesinger et al., 2011; Mack & Kienzle, 2016; Davies et al., 2019].

The clinical response to dietary change, if positive, often occurs within a few days of the introduction of the new diet, but the exclusion diet should still be continued for a minimum of 3-4 weeks as the symptoms could recur [Dandrieux, 2016].

4.2 ANTIMICROBIALS

Antibiotic Responsive Diarrhea (ARD) or Antibiotic Responsive Enteropathy (ARE) are the forms of CIE whose clinical resolution occurs after the introduction of an oral antibiotic therapy [Hall, 2011]. The most used antibiotics for these forms are metronidazole and tylosin. Medium-large sized dogs seem more affected and the German Shepherd Dog (GSD) seems predisposed to this form of CIE. When prolonged antibiotic therapy, usually 4-6 weeks, is suspended the symptoms often relapse, requiring in some cases an extremely prolonged treatment [Allenspach et al., 2007; Dandrieux, 2016].

Until recently, antibiotic therapy was an unavoidable step before proceeding with endoscopy and immunosuppressive therapy [Dandrieux, 2016]. However, the awareness of antibiotic resistance and the increasingly in-depth research in the field of microbiota have supported the idea that antibiotic therapy for CIE is not indicated [Cerquetella et al., 2020]. Recent studies, in fact, show that antibiotic therapies cause profound and long-lasting changes in the intestinal microbiota [Suchodolski et al., 2009; Chaitman et al., 2020; Pilla et al., 2020]. Therefore, the indication is that antibiotic therapy, during CIE, should be used only in cases where it is necessary (eg. Enrofloxacin for HUC) and avoided in other forms of CIE because it could, contrary to what was thought a few years ago, worsen intestinal dysbiosis [Manchester et al., 2019; Cerquetella et al., 2020]. In recent years, the use of probiotics or alternative therapies such as fecal transplantation (FMT) has been increasingly implemented to replace antibiotic therapy.

4.3 IMMUNOSUPPRESSANT

In case of failure to respond to diet and following the execution of an endoscopy of the digestive tract with attached histological examination to investigate the type and severity of inflammation, immunosuppressive therapy should be introduced. The histological evaluation can help the clinician in the choice of the immunosuppressant to use and its dosage, based on the type and severity of inflammatory infiltrate found. After the introduction of immunosuppressive therapy, the clinical response manifests itself in

a short time in 60-80% of subjects, who therefore fall into the IRE group [Craven et al., 2004; Allenspach et al., 2016]. About 15-20% of dogs have no response to immunosuppressive therapy either. These cases, after carefully excluding other pathologies and in particular intestinal lymphoma, are called Non-Responsive Enteropathy (NRE) [Dandrieux, 2016].

The most used immunosuppressive drugs for CIEs are corticosteroids (prednisolone and budesonide), cyclosporine, chlorambucil and azathioprine. Other immunosuppressive drugs (e.g. mycophenolate mofetil) are used in dogs for the treatment of immune-mediated diseases, but the lack of studies on their effects on the GI mucosa does not allow them to be included in the list of drugs for the treatment of IRE [Whitley & Day, 2011; Viviano, 2013; Makielski et al., 2019]. The immunosuppressive drugs most used in the forms of IRE in dogs are briefly described below and doses are reported in Table 4.a.

Prednisolone is usually the drug of choice for moderate to severe inflammatory forms. It has a good clinical response with a low cost, a rapid onset of action (24-48 hours), low side effects and rapidly reversible with dose reduction. Despite the numerous advantages of this drug, there are also disadvantages. Among the first certainly the side effects related to the intake of glucocorticoids which can be difficult to manage especially in large dogs. (≥30kg): polyphagia, polyuria (PU), polydipsia (PD), muscle atrophy, restlessness [Pietra et al., 2013]. Often to achieve good symptom control, a high dosage is required for prolonged periods before it can be reduced. Some subjects have a resistance towards these molecules therefore they do not show a benefit from their intake [Allenspach et al., 2006a]. Finally, they can promote thromboembolic events especially in predisposed dogs (PLE) [Whitley & Day, 2011; Dandrieux, 2016]. Few studies report that budesonide could be a viable substitute for prednisolone as it appears to have a reduced incidence of side effects [Dye et al., 2013; Pietra et al., 2013].

Ciclosporin was initially evaluated as a “rescue” drug for dogs not responding to prednisolone therapy [Allenspach et al., 2006b]. In recent years, its use has spread widely in gastroenterology not only as a "rescue" drug, but also in association with corticosteroids to treat the most serious cases by amplifying the immunosuppressive effect (e.g. PLE) [Allenspach et al., 2016]. This drug is manageable, with low side effects that allow for prolonged therapies. However, it has a very high cost, in the first days of therapy it can induce nausea and vomiting, and it can favor the onset of gingival hyperplasia. It is also recommended to periodically monitor the patient with hematological tests to rule out the onset of myelosuppression, consequent to the action of the drug [Robson, 2003; Whitley& Day, 2011].

Azathioprine is another alternative. This drug has a low cost but takes a long time to become effective, so it is often combined with prednisone in decreasing dosage. Among the side effects are reported liver toxicity, reversible if detected in time, pancreatic and bone marrow toxicity. Frequent monitoring is therefore recommended, especially in the first periods of therapy [Dandrieux et al., 2013].

Chlorambucil is a well-known chemotherapy drug for lymphoma therapy [Lane et al., 2018]. A study has shown that, when combined with prednisone, it seems more effective than azathioprine [Dandrieux et al.,

2013]. It can therefore be a drug to be evaluated in those cases that do not respond to corticosteroid therapy. The main problems related to chlorambucil are the management of animal waste and the bone marrow toxicity it can cause, therefore frequent monitoring is recommended [Whitley& Day, 2011].

There is little indication in the literature on the duration of immunosuppressive therapy in dogs with CIE [Makielski et al., 2019]. In the author's experience, immunosuppressive therapies, in the event of a positive clinical response, should be continued for a prolonged period (several weeks or several months in the most severe cases) and gradually reduced until discontinuation to evaluate a possible relapse of symptoms.

Immunosuppressant	Doses
Prednisone/Prednisolone	1-2 mg/kg every 12h; 20% dosage reduction every 15-20 days
Budesonide	3mg/m^2 every 24h
Cyclosporine	3 to 5 mg/kg PO every 12h or 24h
Azatioprine	1-2 mg/kg PO every 24h
Chlorambucil	2 to 6 mg/m^2 PO every 24h or 48h

Table 4.a: Dosages of the most used immunosuppressive drugs for CIE. Modified from: Sellon, 2013.

PO: oral administration; h: hours

4.4 NUTRIENTS SUPPLEMENTATION

For the malabsorption that afflicts individuals with CIE, they may have various deficiencies, in particular cobalamin, folate, calcium, magnesium, and may also be predisposed to thromboembolic phenomena.

The absorption of cobalamin can be reduced both by a reduction of receptors in the ileum and by a greater consumption by intestinal bacteria, during dysbiosis. In the dog with CIE, a serum cobalamin value <200ng / L was associated with a worse prognosis. The measurement of serum cobalamin is therefore essential to assess the need for supplementation [Allenspach et al., 2007]. The value for which hypocobalaminemia is defined may vary according to the analysis laboratory, in any case the integration is recommended for values lower than 400ng / L. However, it has not yet been proven whether early cobalamin supplementation improves the clinical condition [Kather et al., 2020]. The integration can be orally with daily administration for a period of 12 weeks (50µg/kg/die PO) or parenteral with subcutaneous (SC) administration weekly for the first 6 weeks and then monthly (250-1000 µg/week SC) [Toreson et al., 2016; Kather et al., 2020].

Folate deficiency usually indicates damage to the small intestine receptors, and if associated with cobalamin deficiency it indicates widespread damage. The supplementation is done orally with a dosage of 1-5mg / dog / day [Ruaux, 2013].

Calcium supplementation is recommended in subjects with ionic hypocalcemia (not influenced by the albumin value). In cases of symptomatic hypocalcemia, it is possible to intervene with parenteral administration of 10% calcium gluconate (0.5-1.5mL/kg). In combination, vitamin D can be supplemented orally (0.025-0.06mcg / kg / day) although further studies are needed to confirm this therapy [Whitedead et al., 2015; Allenspach et al., 2017]. Finally, it seems that correcting hypomagnesaemia favors a correction of hypocalcemia (1-2mEq/kg/ day PO) [Kimmel et al., 2000; Cortés & Moses, 2007].

The risk of thromboembolism, especially in dogs with PLE must be considered. In cases where there is a strong predisposition to hypercoagulability (panhypoprotidemia, thrombocytosis, ATIII deficiency, treatment with glucocorticoids) prophylactic therapy for thrombosis should be considered (acetyl salicylic acid 0.5-1mg / kg / day OS or clopidogrel 1-5mg / kg / day OS), although there are no studies to confirm its usefulness in preventing [Goodwin et al., 2011; Sakamoto et al., 2020].

4.5 PRE- AND PROBIOTIC

Probiotics, Prebiotics, Symbiotic, are products used with therapeutic purpose both in acute and chronic enteropathies in dogs. Table 4.b shows the definitions of these products.

DEFINITIONS	
Probiotics	live microorganisms which, if consumed in adequate quantities, confer benefits to the host
Prebiotics	supplements or foods (often dietary fibers or carbohydrates) that selectively stimulate the growth and/or activity of indigenous micro-organisms benefits to the host
Synbiotics	preparations that contain both pre- and probiotics
Postbiotics	nonviable bacterial products that have a beneficial effect on the host

Table 4.b: Key definitions. From: Schmitz, 2020.

Some of the mechanism proposed for probiotics beneficial effects are displacement of pathogenic microorganisms, production of antimicrobial by-products, improvement in GI epithelial barrier function, improvement in micronutrient absorption, and modulation of the enteric and innate immune responses (Figure 4.a) [Thomas & Versalovic, 2010; Schmitz & Suchodolski, 2016]. Among the prebiotic substances there

are fructooligosaccharides (FOS), mannanooligosaccharides (MOS), pectins, inulins and others, which perform their action by acting as a substrate for the intestinal microbiota [Jugan et al., 2017].

Continuous research in the field of intestinal microbiota and the need to limit antibiotic therapy for CIEs as much as possible, in recent years favored the therapeutic use of these substances. Studies on the use of probiotics have evaluated both multi-strain formulations, VSL#3® [Rossi et al, 2014] and Sivomixx® [Rossi et al., 2020b], and single strains, *Enterococcus faecium* [Bybee et al., 2011], *Lactobacillus acidophilus* [Biagi et al., 2007], *Bifidobacterium* sp. *animalis* [Strompfová et al., 2014], *Saccharmyces boulardii* [D'Angelo et al., 2018], with an improvement in the clinical response of the subjects. Studies concerning the beneficial effects of prebiotics have been conducted both as a single supplementation [Nogueira et al., 2019] and in combination with probiotics [Rose et al., 2017; Pilla et al., 2019; Rossi et al., 2020a], demonstrating both clinical and fecal metabolite improvements.

However, few papers are well structured and show a real benefit associated with these products, therefore precise indications on the use of pre-pro-symbiotics cannot be issued. In any case, the use of these products can be associated with therapies for CIE in dogs both to improve the clinical response and to contribute to a lower use of the antibiotics [Schmitz, 2020].

Further attention in employing probiotics is represented by the real content in lyophilized tablets and the bacterial viability, because some studies has demonstrated that the indicated content of probiotic often do not correspond with the real it [Gaspardo et al., 2020].

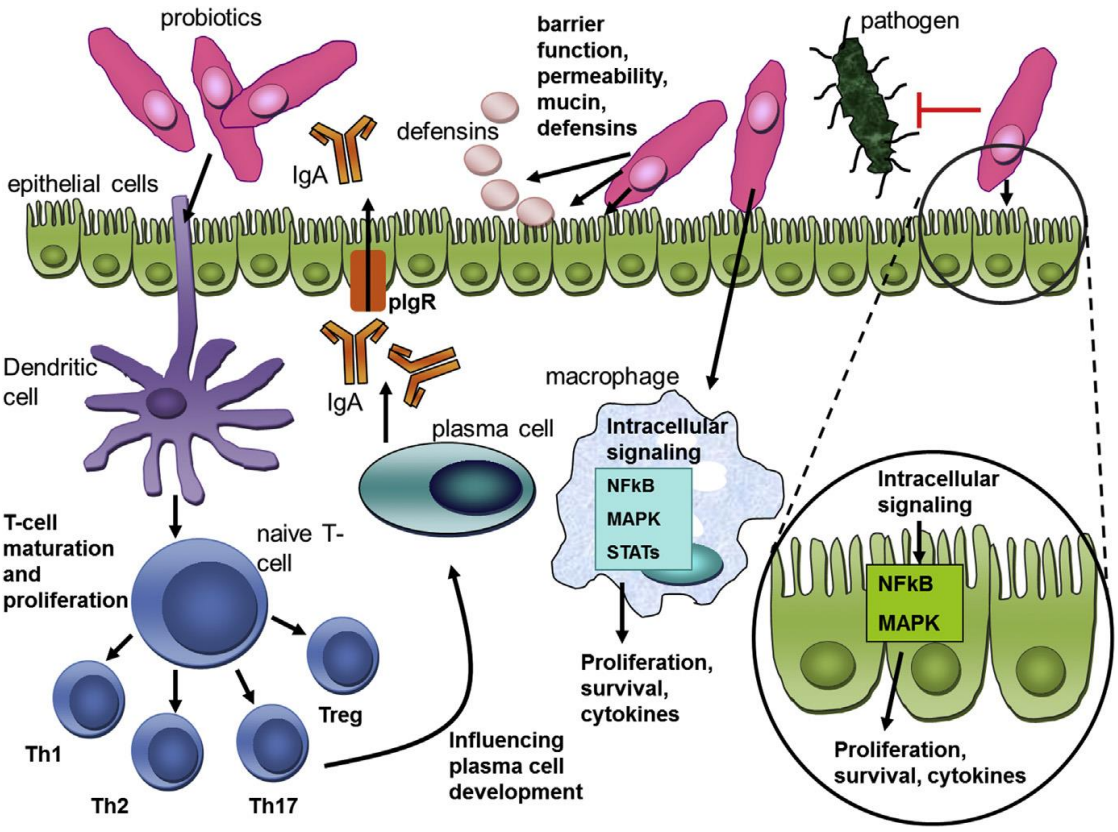

Figure 4.a: Proposed mechanisms of action of probiotics. From Schmitz & Suchodolski, 2016.

4.6 FECAL MICROBIOTA TRANSPLANTATION

Fecal Microbiota Transplantation (FMT) refers to the procedure whereby fecal material, collected from a chosen healthy donor, is delivered to a patient via different routes of administration. It is an established technique in human medicine for several uses, such as infections resistant to *Clostridium difficile* [Cammarota et al., 2014; Singh et al., 2014]. In addition to other pathologies, it has also been applied in human patients with IBD with positive results [Colman & Rubin, 2014], thus favoring its use also in veterinary medicine [Bottero et al., 2017].

Donor selection in veterinary medicine is not standardized. To be chosen, a donor must not have concomitant pathologies (clinical history and basic general examinations), should not have taken antibiotic therapies in the previous 3 months, and, according to some authors, it is preferable to eat a controlled diet with a reduced number of ingredients [Chaitman et al., 2016; Chiatman & Gaschen., 2020].

The patient with CIE to undergo FMT (recipient) must be selected after carefully excluding all extra GI causes. It is not recommended to associate FMT with antibiotic therapy, as it could nullify the beneficial effect of FMT, while there does not seem to be any contraindications to associate it with immunosuppressive therapy, with the advice of closely monitoring the patient [Chiatman & Gaschen., 2020].

There are several techniques of preparation of the fecal sample, usually it is homogenized and filtered, to remove particulates, and subsequently it can be administered fresh or frozen. Also, for the routes of administration there are different methods both by oral route (endoscopy, naso-duodenal probe, oral capsules) and rectally (by enema or colonoscopy) [Chaitman et al., 2016].

Studies have shown that in dogs with CIE that did not have an adequate clinical response with standard therapies (diet, antibiotics, immunosuppressants), obtained improvement following treatment with FMT [Bottero et al., 2017; Niina et al., 2019]. In dogs with acute diarrhea, treatment with metronidazole or FMT resulted in a similar clinical recovery, however those treated with metronidazole had persistent changes in fecal microbial and metabolic profile, suggesting a possible use of FMT even in cases of acute GI symptoms [Chaitman et al., 2020].

Studies on fecal transplantation in dogs with CIE are still few and non-standard, but the easy administration and the promising results obtained so far are a stimulus to expand the use of FMT in dogs.

4.7 THERAPIES IN DEVELOPMENT

In humans, bile acid malabsorption (BA) is well known, although underestimated, as a cause of chronic diarrhea. The therapy of choice in humans is cholestyramine, although its use is limited due to its palatability and interactions with other drugs. Glucocorticoids and a low-fat diet can reduce the secretion of bile acids, with several mechanisms [Wilcox et al., 2014]. In dogs there are few studies regarding BA malabsorption diarrhea, therefore it is not recommended to start therapy, also because normal therapies (low-fat diet and corticosteroids) may already improve this pathology [Kent et al., 2016; Blake et al., 2019].

The use of stem cells as a therapy for IBD in humans has given promising results, thus stimulating research also in veterinary medicine [Mao et al., 2017]. For current knowledge, there is only one study in the dog [Pérez-Merino et al., 2016], and one in the cat [Webb & Webb, 2015], for the use of stem cells during CIE. Despite the positive results of these studies, further evaluation is certainly needed before considering stem cells an adequate therapy.

4.8 PROGNOSIS

The prognosis in dogs with CIE is highly variable. It appears more favorable in dogs with the FRE form, with a faster and more lasting symptom remission (years), than in those with IRE, which usually show a clinical response after months and fluctuating or relapsing symptoms. Dogs with PLE seem to be those with the poorest prognosis (45 days to 5 months), due to the severity of the clinical findings and the refractoriness to treatments, although some dogs with PLE manage to have long survival times [Allenspach et al., 2007; Volkmann et al., 2017].

The negative prognostic factors that can help in understanding the severity of a clinical case and thus issuing a prognosis are summarized in the Table 4.c.

Factor	References
CIBDAI and CCECAI	Allenspach et al., 2007; Gianella et al., 2017; Kathrani et al., 2019.
Hypoalbuminemia	Craven et al., 2004; Allenspach et al., 2007.
Hypocobalaminemia	Allenspach et al., 2007; Steiner, 2014.
CRP	Allenspach et al., 2007; Steiner, 2014.
cPLI	Kathrani et al., 2009.
Urea	Kathrani et al., 2019.
Hypovitaminosis D	Allensapch et al., 2017.

Table 4.c: Negative prognostic factors in dogs with CIE.

The type of CIE of the patient under examination, associated with the clinical condition, the clinical scores and the collection of data considered negative prognostic factors, help the clinician to understand the severity of the case under examination and to issue a prognosis. The clinical conditions associated with a worse prognosis are discussed in chapter 2.2.

INFLUENCE OF LACTOBACILLUS KEFIRI ON INTESTINAL MICROBIOTA AND FECAL IGA CONTENT IN HEALTHY DOGS

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ABSTRACT

The increasing incidence of gastrointestinal tract pathologies in dogs and the worrisome topic of antibiotic resistance have raised the need to look for new therapeutic frontiers. Of these, the use of probiotics represents a potential therapeutic alternative. *Lactobacillus kefiri* (*Lk*) is a species of *Lactobacillus* isolated from kefir. Previous studies have demonstrated that its administration in mice downregulates the expression of proinflammatory mediators and increases anti-inflammatory molecules in the gut immune system. It also regulates intestinal homeostasis, incrementing immunoglobulin A (IgA) secretion. Since *Lk* has never been studied as a single probiotic in dogs, the aim of this study was to evaluate the safety of *Lk* in dogs, and its effect on IgA secretion and on intestinal microbiota composition. Ten healthy dogs without a history of gastrointestinal diseases were included. The dogs received *Lk* at a dose of 10^7 live microorganisms orally, once daily for 30 days. The fecal samples were tested before administration, in the middle, at the end, and 30 days after discontinuation. The IgA secretion concentration and the microbiota composition were evaluated on the fecal samples. The results in this study suggested that *Lk* did not influence the concentration of IgA, nor significant changes of the intestinal microbiota were observed during and after the treatment. Therefore, additional studies are needed to investigate if a higher daily dosage of *Lk* can influence the intestinal homeostasis of dogs.

Key words: dog, stool, gut microbiota, probiotic, IgA

INTRODUCTION

In recent years, intestinal microbiota has become increasingly relevant for veterinary scientists and has been studied for its role on the welfare of the host (1). It is an ecosystem including mainly bacteria, but also archaea, fungi, protozoa, and viruses, which plays several roles in the host physiology by means of a range of metabolic and immunological interactions. In fact, this complex ecosystem helps in the digestion of food by assisting the absorption and metabolism of nutrients, and has trophic and protective functions (2). It defends the gastrointestinal tract (GIT) against pathogenic organisms, promotes mucus production and enterocyte turnover, and modulates host immune development and functionality (3). In particular, commensal bacteria provide intestinal immune protection by regulating, among other things, the secretion of IgA, the lack of which seems to be correlated with chronic enteropathies in dogs (4, 5).

Canine chronic enteropathies, categorized into four classes (food responsive; antibiotic responsive; immunosuppressant responsive; nonresponsive enteropathy) according to the response to treatment, are multifactorial diseases where host genetic factors, the immune system, and indigenous intestinal bacteria are supposed to be engaged in intricate interactions (6, 7).

Dogs affected by antibiotic responsive enteropathy (ARE) are generally young, predominantly belong to large breeds, and show remission of clinical signs following antimicrobial administration (metronidazole, tylosin, doxycycline, rifaximine) (8, 9). It is thought that antimicrobials are able to modify the intestinal microbial population by counteracting its imbalances (i.e., dysbiosis); however, although a short-term response to metronidazole and tylosin has been reported, very few papers have described the long-term control of ARE (9–11).

Moreover, there is now evidence that prolonged treatment with antibiotics, particularly with metronidazole, can lead to permanent unfavorable changes in the microbiota, promoting antimicrobial resistance, currently one of the most important threats to public health (12, 13).

Therefore, the increasing incidence of GIT pathologies and the worrisome topic of antibiotic resistance create the need for new therapeutic options (14), and toward replacing antibiotics with (a) probiotics (live microorganisms that confer a healthy benefit on the host); (b) prebiotics (a substrate selectively utilized by host microorganisms useful for reestablishing a eubiotic microbiota layout); (c) synbiotics (a mixture of probiotics and prebiotics having a synergistic action on host health); and (d) postbiotics (soluble factors or metabolic byproducts, secreted by live bacteria or released after bacterial lysis expressing biological activity in the host).

Lactic acid bacteria (LAB), including *Lactococcus, Streptococcus, Enterococcus, Pediococcus, Leuconostoc*, and *Lactobacillus,* are probiotic bacteria that are normally part of the intestinal microbiota of dogs and cats (2). *Lactobacillus* species are distributed throughout the canine intestinal tract in varying amounts (2). Several strains of Lactobacilli have specifically been studied for their ability to reduce the number of pathogenic bacteria in the canine intestine (15–20).

Among different naturally fermented foods and their potential probiotic properties, particular attention has recently been focused on kefir, a dairy product that could modulate canine intestinal microbiota if given regularly (21). Kefir has a complex composition of microbial organisms, which includes several species of LAB as well as acetic acid bacteria and yeast (22). *Lactobacillus kefiri* (*Lk*) is a *Lactobacillus* species that has been isolated from kefir (23). Previous studies have demonstrated that its administration in mice downregulates the expression of proinflammatory mediators and increases anti-inflammatory molecules in the gut immune system (24, 25). It also regulates intestinal homeostasis by increasing IgA secretion and mucin production (25). Probiotic properties of *Lk* have also recently been demonstrated in humans (26); however, to the best of the authors' knowledge, the use of *Lk* has never been evaluated in dogs.

The aim of this study was to evaluate the safety and ease of administration of *Lk* in healthy dogs and its ability to impact the intestinal microbiota composition and IgA secretion.

MATERIALS AND METHODS

Lactobacillus kefiri Administration

This study is based on the use of a commercial food supplement (Kefibios§R), provided by the company Hulka S.r.L. (Rovigo, Italy), containing live lactic ferments of *Lk* (LKF01–DSM 32079), currently used as probiotics in human medicine. Copyright permission to publish the product name (Kefibios§R) was given by the Chief Executive Officer of Hulka S.r.L. The product is marketed in capsules. Following the label of the product, five

drops of the solution reconstituted with 6 ml of vegetable oil in prefilled bottles contain $\geq 10^9$ active fluorescent units (AFUs) of live and viable *Lk* (ISO 19344:2015). The dose corresponds to the human one indicated by the company, regardless of age and body weight (BW). The study, involving 10 healthy privately owned dogs, was conducted at the Veterinary Teaching Hospital (VTH) of our department. The owners were carefully instructed as to how to use and mix the product, and then shake the bottle before each administration. The product was then stored at room temperature between 10°C and 25°C and away from direct light.

The recruitment of the dogs in the study was voluntary and at no cost to the owners. Written informed consent before enrollment in the study was obtained from the owners.

The trial was authorized by the Animal Welfare Committee of the University of Bologna (Protocol No. 3885 of 21/07/2017).

Animals and Experimental Design

Privately owned dogs of various breeds, genders, and weight, over 1 year of age, were enrolled in the trial. Dogs with any disease in the previous 2 months before the start of the trial were excluded. The inclusion criterion was the absence of antimicrobial or immunosuppressive treatment up to 60 days prior to the start of

and during the trial. For inclusion, each dog was evaluated with a clinical examination and a laboratory panel, which included a complete blood count, a serum biochemistry profile, and coprological examination for gastrointestinal parasites.

The body condition score (BCS) was calculated using the 1– 9 score proposed by Royal Canine SAS, and the fecal score was evaluated according to the Fecal Score System (FSS 1-7) proposed by Nestlé-Purina Petcare. The BCS was determined as follows: 1–3 = too thin, 4–5 = ideal, and 6–9 = too heavy. The fecal samples were scored as follows: 1 = very hard and dry, leaves no residue on the ground when picked up; 2 = firm, but not hard, pliable, little or no residue on the ground when picked up; 3 = log-shaped, moist surface, leaves residue on the ground, but holds form when picked up; 4 = very moist and soggy, leaves residue on the ground and loses form when picked up; 5 = very moist but has a distinct shape, leaves residue on the ground and loses form when picked up; 6 = has texture, but no defined shape, leaves residue on the ground when picked up; and 7 = watery, no texture, present in flat patches.

Not being experimental animals but privately owned dogs, diet was not standardized during the trial but the previous diet (Table 1) was maintained, and water was supplied *ad libitum*.

During the entire experimental time, the dogs received five drops (≥10^9 AFUs) of *Lk* once daily for 30 days, administered directly in the mouth during the dinner meal.

Clinical examination, BCS, and FSS were performed at inclusion, and, during the trial, respectively, 15, 30, and 60 days after the start of *Lk* administration.

Samples of fresh feces were collected from each dog on three consecutive days: (a) at T0, before the start of *Lk* administration (days −3, −2, −1); (b) at T15, in the intermediate time of *Lk* administration (days 13, 14, 15); (c) at T30, at the end of *Lk* administration (days 28, 29, 30); (d) at T60, 1 month after the last *Lk* administration (days 58, 59, 60).

The samples were collected by the owner immediately after defecation, immediately stored at −20°C in the owner's household freezer until delivered frozen to the department where they were stored at −80°C until use.

All the samples were analyzed for IgA detection, while, for the gut microbiota, a pool of three samples from each dog at T0, T30, and T60 was prepared and analyzed.

Dogs	Breed	Gender	Age	Weight	BCS	Fecal score	Diet
1	Mix breed	C	4y1m	33 kg	6	2	Purina tonus dog chow chicken
2	Mix breed	S	4y0m	18.3 kg	6	2	Purina tonus dog chow chicken
3	Border Collie	F	2y6m	19 kg	4	2	Prolife adult medium chicken and rice
4	Australian shepherd	S	6y0m	23 kg	5	2	Royal canin veterinary diet neutered adult medium dog
5	Border Collie	C	2y6m	20.5 kg	5	2	Farmina ancestral low grain lamb and blueberry
6	Dachshund	F	3y4m	5.6 kg	6	2	Royal canin small dog chicken and rice
7	Border Collie	S	9y7m	19 kg	5	2	Prolife adult medium chicken and rice
8	Labrador retriever	M	2y5m	32.1 kg	5	3	Monge natural superpremium rabbit, rice, and potatoes
9	Mix breed	S	9y8m	7.5 kg	5	3	Royal canin small dog chicken and rice
10	Mix breed	C	5y11m	5.7 kg	5	2	Royal canin small dog chicken and rice

Table 1: Dogs included in the study.

M, male; C, neutered male; F, female; S, neutered female; BCS, body condition score

Fecal Microbiota Analysis

Microbial DNA was extracted from about 250 mg of pooled fecal sample, derived from the experimental points (T0; T30; T60) for each dog, using the repeated bead-beating plus column method, as previously described (27). Briefly, samples were suspended in 1 ml of lysis buffer (500 mM NaCl, 50 mM Tris-HCl, pH 8, 50 mM EDTA, and 4% SDS) and bead-beaten three times in a FastPrep instrument (MP Biomedicals, Irvine, CA, USA) at 5.5 movements/s for 1 min, in the presence of four 3-mm glass beads and 0.5 g of 0.1-mm zirconia beads (BioSpec Products, Bartlesville, OK, USA). After incubation at 95˚C for 15 min, samples were centrifuged at 13,000 rpm for 5 min. Two hundred and sixty microliters of 10 M ammonium acetate was added to the supernatant, followed by 5-min incubation on ice and 10- min centrifugation at 13,000 rpm. The supernatant was added with one volume of isopropanol, followed by incubation on ice for 30 min. Precipitated nucleic acids were washed with 70% ethanol, resuspended in 100 µl of TE buffer, and treated with 2 µl of 10 mg/ml DNase-free RNase at 37˚C for 15 min. DNA was further purified using the QIAamp Mini Spin columns (QIAGEN, Hilden, Germany) following the manufacturer's instructions. DNA concentration and quality were evaluated using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

The V3–V4 hypervariable region of the 16S rRNA gene was amplified by using the 341F and 785R primers with Illumina adapter overhang sequences, as previously described (28). PCRs were performed in a final volume of 25 µl, containing 12.5 ng of genomic DNA, 200 nM of each primer, and 2X KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Wilmington, MA, USA), in a Thermal Cycler T (Biometra GmbH, Göttingen, DE) with the following thermal cycle: initial denaturation at 95˚C for 3 min, 25 cycles of denaturation at 95˚C for 30 s, annealing at 55˚C for 30 s and extension at 72˚C for 30 s, and a final extension step at 72˚C for 5 min. Amplicons were purified using a magnetic bead-based system (Agencourt AMPure XP; Beckman Coulter, Brea, CA, USA). Indexed libraries were prepared by limited-cycle PCR using Nextera technology and further purified as described above. Final libraries were pooled at equimolar concentration, denatured with 0.2 N NaOH, and

diluted to 6 pM before loading onto the MiSeq flow cell. Sequencing was performed on Illumina MiSeq platform with a 2 × 250 bp paired-end protocol, according to the manufacturer's instructions (Illumina, San Diego, CA, USA).

Sequencing Reads Were Deposited in the National Center for Biotechnology Information Sequence Read Archive (NCBI SRA; BioProject ID PRJNA 592436).

Quantification of IgA Fecal Content

Stool samples were thawed and subsequently freeze-dried (Modulyo EF4, 1044, Edwards, Apeldoorn, The Netherlands) for 16 h in order to eliminate the water contained and standardize the subsequent analysis, as reported by Grellet et al. (29). The lyophilized fecal samples were resuspended in 1X PBS (phosphate buffer saline) containing 0.5% Tween20 (Sigma- Aldrich, St. Louis, MO, USA), according to a weight/volume ratio of 100 mg/ml (1:10 dilution) by vortex (3') and then centrifuged at 1,500 × *g* for 10 min. After removing the supernatant, a further centrifugation was carried out at 10,000 × *g* for 20 min, then the aqueous phase was taken and frozen at −20°C until the processing moment. The determination of the IgA secretion amount was carried out by using a commercial kit (dog IgA ELISA Quantitation Set, Bethyl Laboratories Inc., TX, USA; Assay Range: 15.6–1,000 ng/ml), following the manufacturer's protocol.

Kefibios® Quality Control

In order to confirm the content of *Lk* declared by the manufacturer, we performed an independent assessment of *Lk* concentration in Kefibios[§]R capsules (Bacteriological Laboratory, UNI EN ISO 9001:2015 registration number IT-15164).

Five different batches of the product, acquired in several drugstores, with expiration date from 1 to 2 years with respect to the time of the analysis, were analyzed, and enumeration of viable bacteria was conducted by the plate count method.

Briefly, each sample was firstly solubilized in MRS Broth (Oxoid CM359, Thermo Scientific, MA, USA) and gently shaken at room temperature for 15 min by using an orbital shaker, then serially diluted in the same broth and inoculated onto MRS Agar (Oxoid CM361, Thermo Scientific, MA, USA) plates. Plating was performed in triplicate. Plates were incubated at 37°C for 72 h under anaerobic atmosphere, and the number of colony-forming units (CFUs) was determined. The colonies obtained in the tests were identified by the API 50 CHL (BioMerieux, FI, Italy) test, and results are presented as the number of viable cells per capsule and per dose (five drops).

Bioinformatics and Statistics

For the analysis of fecal microbiota composition and diversity, raw sequences were processed using a pipeline combining PANDAseq (30) and QIIME 1 (31). High-quality reads were binned into operational taxonomic units (OTUs) through an open-reference strategy at 0.97 similarity threshold by using UCLUST (32).

Taxonomy was assigned using the RDP classifier and Greengenes as a reference database (release May 2013). All singleton OTUs and chimeras, identified by ChimeraSlayer (33), were discarded. Alpha rarefaction was performed using observed OTUs, Shannon, and the phylogenetic diversity (PD) whole tree metrics, while beta diversity was estimated by computing weighted and unweighted UniFrac distances. For the identification of *Lk*, OTUs assigned to the genus *Lactobacillus* were subjected to BLAST analysis (34).

All statistical analyses were performed in R (version 3.1.3) using the packages vegan, made4, and GraphPad Prism V.5.01 (GraphPad Software, La Jolla, CA, USA).

Assessment of data for normality was carried out by applying the D'Agostino and Pearson Omnibus normality test.

UniFrac distances were used for principal coordinates analysis (PCoA), and the significance of data separation was tested using a permutation test with pseudo-F ratios (function Adonis of vegan). Wilcoxon test for paired data was used to assess significant differences in alpha diversity and taxon relative abundance between groups.

A repeated-measures ANOVA (with Tukey *post hoc* test) was applied to evaluate the differences in BW and fecal IgA content between experimental points (T0, T15, T30, and T60). Friedman test (with Dunn's as *post hoc* test) was applied to evaluate the differences in BCS and FSS between experimental points (T0, T15, T30, and T60).

$p < 0.05$ was considered statistically significant.

RESULTS

Animals

Ten healthy adult dogs were included. Of those, one was male, three neutered males, two females, and four neutered females. Mean age was 4.9 ± 2.8 years (range 2–9). Breeds included were mixed breed dogs (*n* = 4), Border Collies (*n* = 3), Labrador Retriever (*n* = 1), Australian Shepherd (*n* = 1), and Dachshund (*n* = 1). Their BW ranged from 5 to 33 kg (18.3 ± 9.84 kg), while their average BCS was normal (range 4–6). Initial FSS was normal in all dogs (range 2–3; Table 1). All dogs had normal hematological and biochemical parameters, and the coprological examination was negative for parasites. The liquid product containing *Lk* was spontaneously accepted by all the subjects. No clinical signs during the trial (with the exception of dog #6), and up to 1 month later, were reported by the owners. There were no significant changes in BW and BCS during the study period, nor did the FSS of each animal changed. Dog #6 developed a urinary tract infection at day 50 of the trial that required antibiotic treatment. This dog's last fecal sample was therefore excluded from the analysis.

Microbiota Analysis

A total of 1,536,903 high-quality reads (mean ± SD, 52,997 ± 16,302) were obtained and clustered into 2,716 OTUs at 97% similarity. The PCoA of intersample diversity based on weighted and unweighted UniFrac distances showed no significant separation among the study groups (i.e., baseline, end of treatment, and follow-up; $P = 0.7$, permutation test with pseudo-F ratios; Figure 1A). Similarly, no significant differences were found in alpha diversity after *Lk* administration or in the follow-up compared to the baseline ($P > 0.05$, Wilcoxon test; Figure 1B).

In line with the available literature on the gut microbiota of healthy dogs (35, 36), the phylum-level microbial profiles at the baseline were dominated by Firmicutes (relative abundance, mean±SEM,73.1±4.2%), with Actinobacteria (14.2 ± 3.1%), Bacteroidetes (7.8 ± 2.2%), and Fusobacteria (3.9 ± 1.2%) as minor components (Figure 2). *Lachnospiraceae*, *Coriobacteriaceae*, *Clostridiaceae*, and *Erysipelotrichaceae* were the major families of the baseline microbiota (relative abundance ≥ 10%; Figure 3A). Consistently, the most represented genera were *Blautia*, *Clostridium*, and *Collinsella* (Figure 3B). Following *Lk* administration, no significant differences in taxon relative abundance at these phylogenetic levels (i.e., phylum, family, and genus) were observed (Figures 2, 3). One month after the end of the treatment, these changes were no longer detectable.

However, at T60, a decreasing trend in the relative abundances of *Fusobacteriaceae* and *Ruminococcaceae* was found (T0 vs. T60, $P = 0.11$ and 0.15, respectively; Figure 3A). With specific regard to *Lk*, OTUs assigned to this species were not present at T0 but accounted for 10.3% of *Lactobacillus* diversity (and 0.07% of the intestinal ecosystem) after 30 days of *Lk* administration, which then disappear again in the follow-up.

IgA Content in Fecal Samples

A total of 117 fecal samples were taken and analyzed. Three samples (T58, T59, and T60 from dog #6) were missing because dog #6 was excluded from the trial.

The water content of feces was very similar among all the samples with percentage values of 38.08 ± 4.97 (mean ± SD).

IgA was detected in all samples except for sample T15 from dog #3 (undetectable < 15.6 ng/ml). All dogs have shown a huge intra-individual variability of fecal IgA content among the three following day samples at T0, T15, T30, and T60 (Figure 4A).

Only in four dogs it was possible to observe an increasing trend in the IgA fecal content (dogs 1, 2, 3, and 6; Figure 4A).

In Figure 4B, we showed a ratio between the quantity of IgA fecal content at T15, T30, and T60 and the basal (T0) for each dog.

Overall, the fecal IgA content between different time points did not show any significant differences (p = 0.1; Figure 5).

Kefibios® Quality Control

The number of viable *Lk* per capsule from different product batches varied from 7.73 × 10^7 CFU to 152 × 10^7 CFU (81 ± 61 × 10^7 CFU, mean and standard deviation). The number of viable *Lk* in the liquid formulation per dose of five drops varied from 0.39 × 10^7 CFU to 7.55 × 10^7 CFU (3.2 ± 2.4 × 10^7 CFU, mean and standard deviation) and was hence 2-log fold lower than the declared concentration (≥10^9 CFU) by the company.

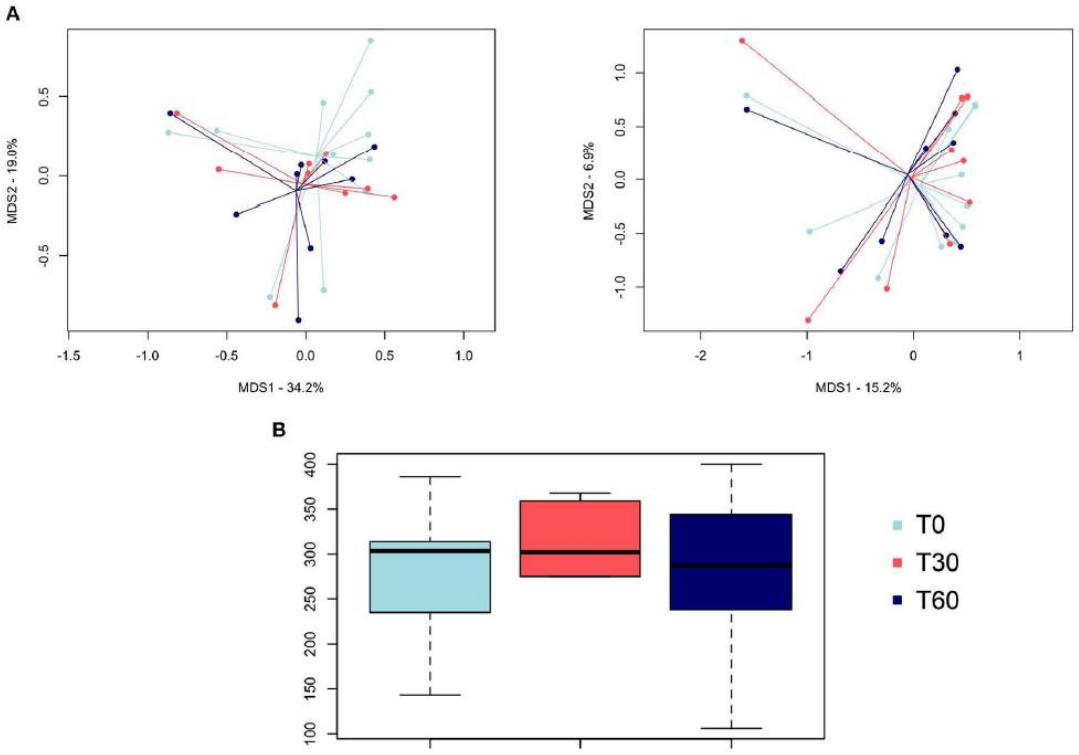

Figure 1: Gut microbiota diversity of healthy dogs following *Lk* administration. (A) Principal coordinates analysis of intersample diversity, based on weighted (left) and unweighted (right) UniFrac distances. (B) Alpha diversity computed with observed OTU metrics. T0, baseline; T30, after 30 days of *Lk* administration; T60, 1 month after the end of the treatment.

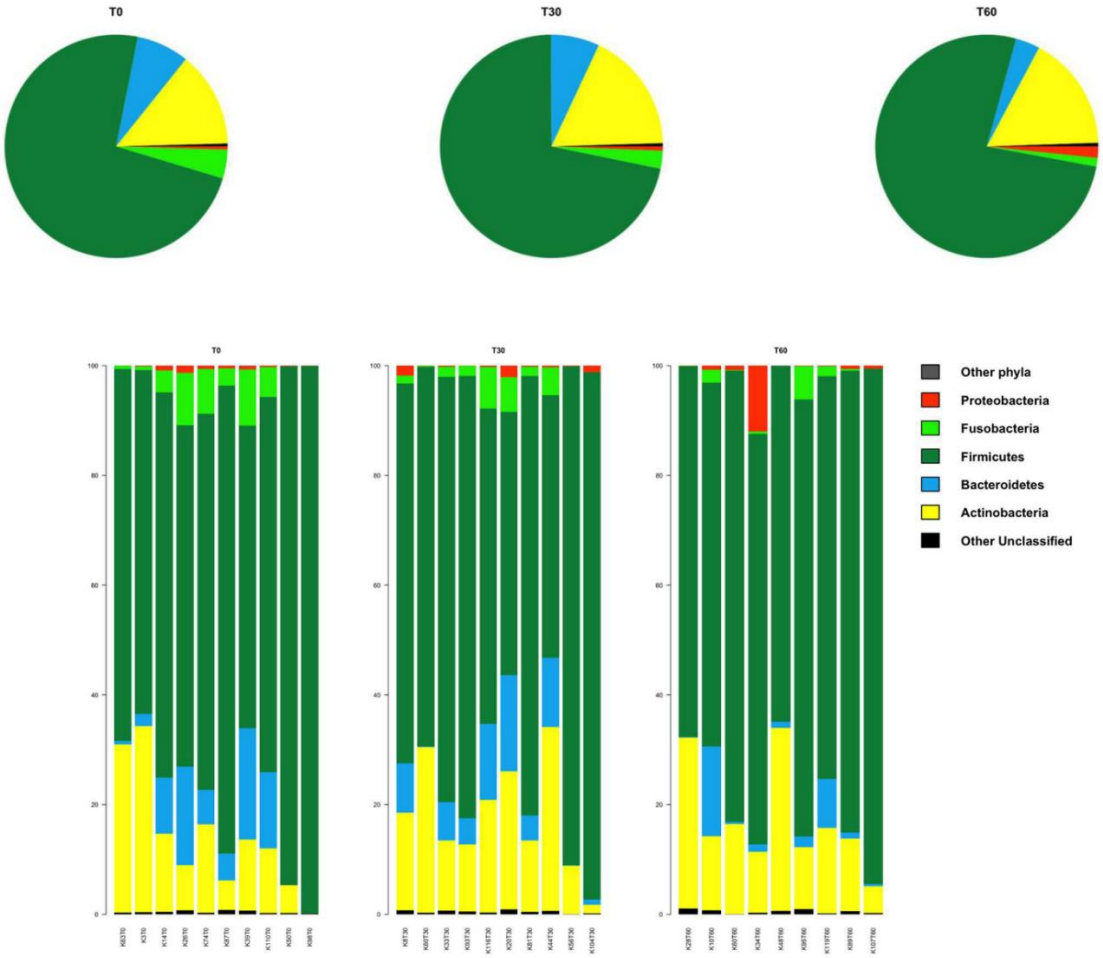

Figure 2: Impact of *Lk* administration on the phylum-level compositional structure of the gut microbiota of healthy dogs. Top: pie charts of mean values of relative abundance; Bottom: bar plots of individual profiles. T0, baseline; T30, after 30 days of *Lk* administration; T60, 1 month after the end of the treatment

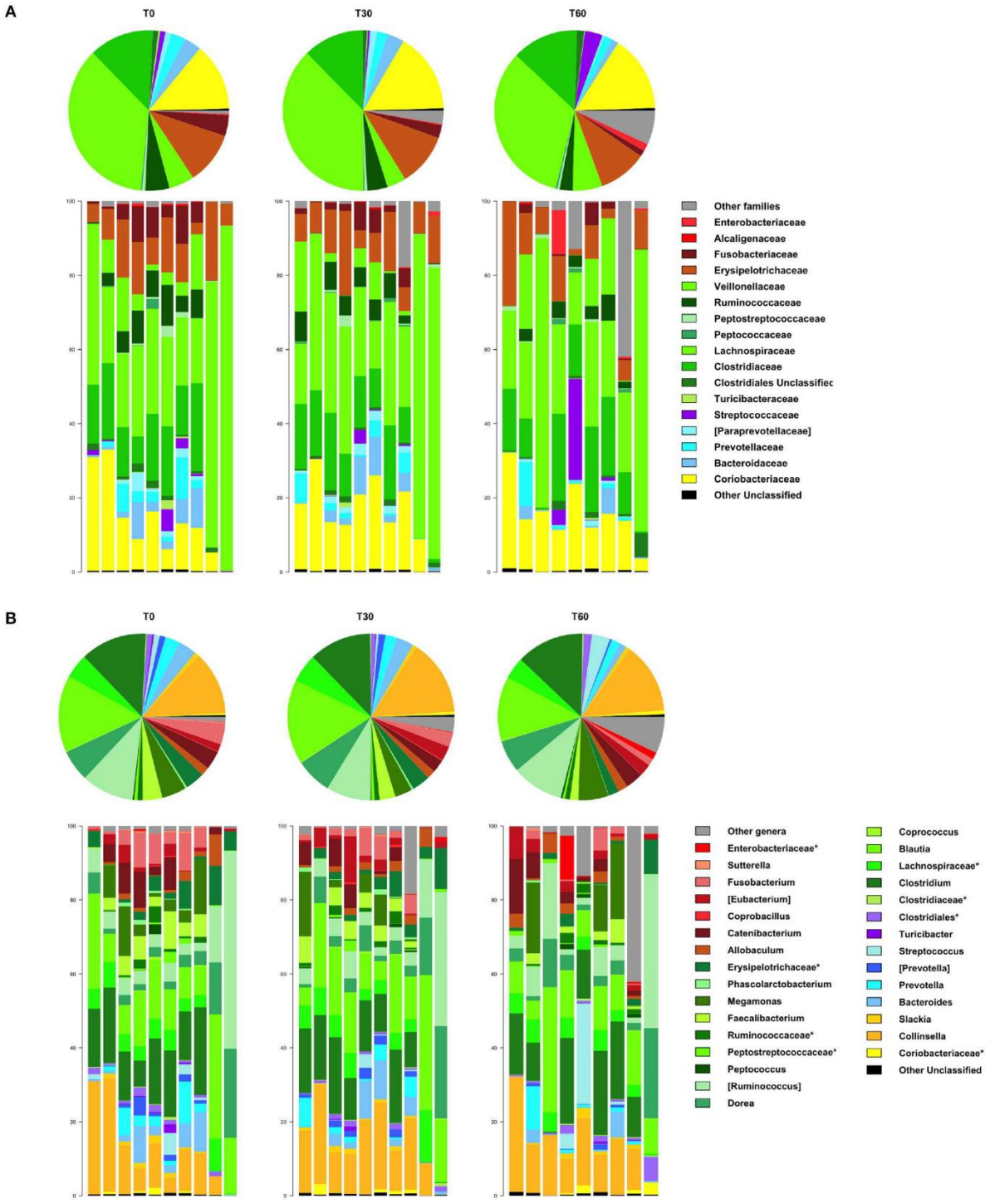

Figure 3: Impact of *Lk* administration on the phylum-level compositional structure of the gut microbiota of healthy dogs. Top: pie charts of mean values of relative abundance; Bottom: bar plots of individual profiles. T0, baseline; T30, after 30 days of *Lk* administration; T60, 1 month after the end of the treatment

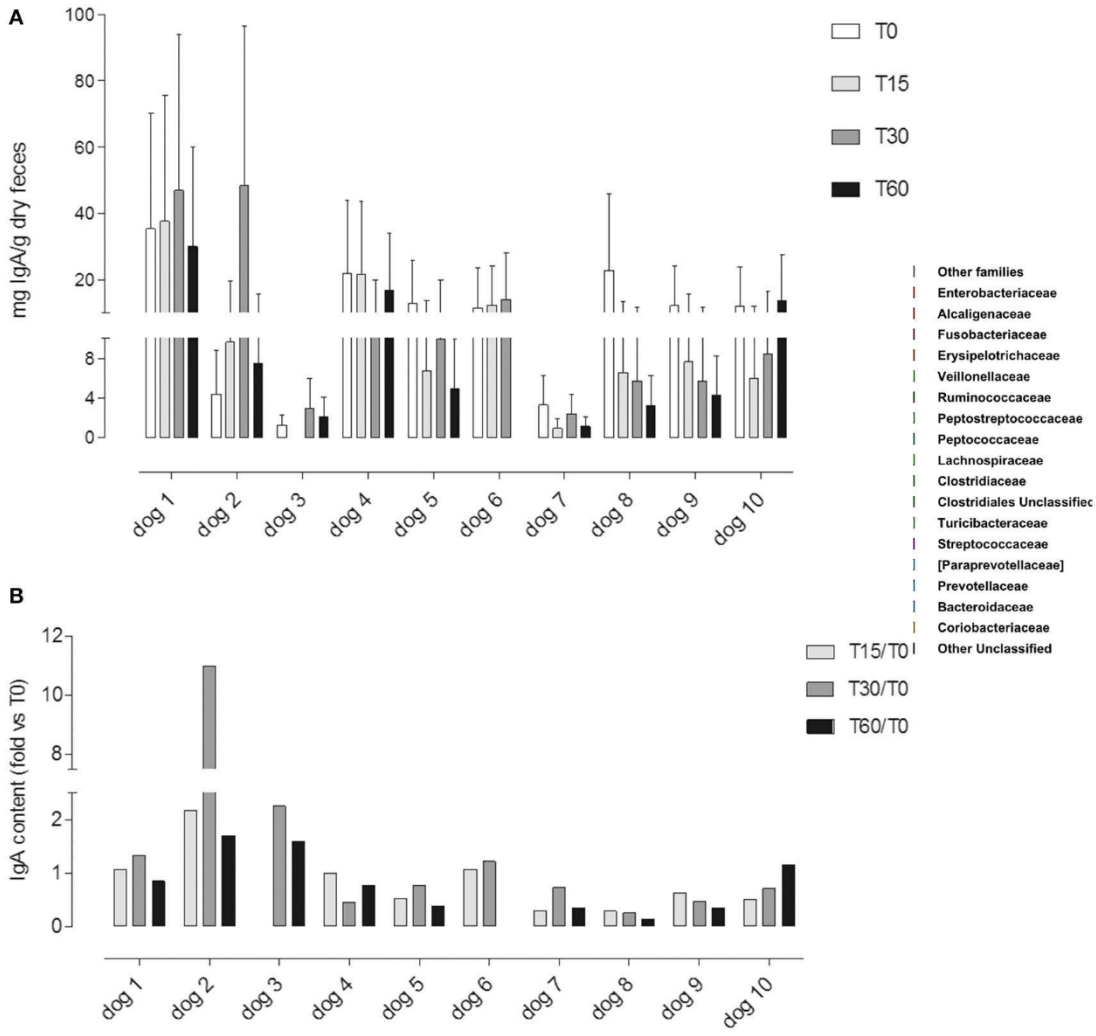

Figure 4: (A) Fecal IgA content at different time points (T0; T15; T30; T60); each value (mean ± SD) represents the average of the measurements of the three samples collected in three consecutive days for each experimental point: T0, before the administration, T15 and T30, after 15 and 30 days of *Lk* administration; and T60, 1 month after the last *Lk* administration. The T60 fecal samples of dog #6 were excluded from the analysis due to antibiotic therapy. (B) IgA fecal content evaluated by the ratio between the quantity at T15, T30, and T60 and the basal (T0) for each dog.

DISCUSSION

In recent years, interest in characterizing the canine intestinal microbiota has soared, and therapeutical interventions that can positively influence the microbiota composition and function, specifically identification of novel probiotics, are sought (2, 3, 37). Several potential probiotics have already been tested in dogs, including bacterial species belonging to the genera *Enterococcus*, *Lactobacillus, Bifidobacterium*, and *Saccharomyces*, demonstrating their role in the treatment of acute and chronic enteropathies (15–18, 38–40).

Among *Lactobacillus* strain, several species have been already studied (*Lactobacillus acidophilus* DSM13241; *Lactobacillus fermentum* CCM7421; *Lactobacillus animalis* LA4). However, the achieved results

are difficult to compare as different dosages (10^7 CFU/daily dose; 10^9 CFU/daily dose; 3 to 3.6 × 10^9 CFU/daily dose) and different forms of application were used, and the duration of administration was also variable (15, 17, 18).

To date, no studies have evaluated the influence of *Lk* on the parameters of intestinal health in dogs. Only one previous study has assessed the effect of kefir (a fermented dairy product containing *Lk*) on dogs (21). This study demonstrated a significant increase in the fecal LAB:*Enterobacteriaceae* ratio and a decrease in the fecal Firmicutes:Bacteroidetes ratio, which was interpreted as an improvement of the gut microbiota composition. However, this study did not use a single bacterial strain, but the mixture of more than 50 microorganisms contained in kefir, and only reported a dose for total LAB (9.32 ± 0.23 $\log_{10}$ CFU/ml) and yeast (7.12 ± 0.36 $\log_{10}$ CFU/ml) (21). It is therefore difficult to calculate and compare the precise concentration of *Lk* that was administered in this study, and to infer the changes observed to a single potentially probiotic strain, as it could be attributed to several microorganisms and their potential synergism.

In addition, the composition of microorganisms in kefir may vary depending on its origin, the substrate used in the fermentation process, and the culture maintenance methods (41). The product used in our study is a commercial preparation registered as a probiotic for human medicine, containing *Lk* with 30-day stability of reconstituted product guaranteed by the producer. Currently, there are no recommendations for the dosage of *Lk* in dogs. Daily dosages used in mice and people were 10^8 and 10^{10} CFU, respectively (24, 26).

The dose administered to dogs in the present study was extrapolated from the dose for an adult person as recommended by the manufacturer. While the daily dose for people (five drops) should contain ≥10^9 AFUs of live and viable *Lk*, the results presented here indicate that the same dose, administered to the dogs of this study, was more equal to 3.2 ± 2.4 × 10^7 CFU.

The 30-day duration of the experiment was chosen based on available literature and a suspected washout time of 4 weeks after discontinuation of administration (42).

Fecal samples were chosen over other types of samples to evaluate the intestinal microbiota, as they can be collected in a noninvasive manner, raising no ethical concerns in comparison to, for example, intestinal mucosal biopsies. In addition, the ability of *Lk* to modulate microbiota composition has already been demonstrated using fecal samples in people and mice (25, 26). Similarly, concentrations of IgA from duodenal biopsies and fecal samples showed no difference in a previous study (4).

As for the gut microbiota, in line with what has already been reported for probiotic supplementations in healthy individuals (43), no changes were detected after *Lk* administration.

The IgA results showed a large interdog variability at T0 (from 1.33 to 35.35 mg/g of dehydrated feces), which, although all dogs appeared clinically healthy, could depend on the extreme variability in experimental dogs' signaling, age, life environment, and food taken.

Moreover, our IgA variability is in agreement with the data reported by other authors (29, 44).

Regardless of the basal value, we have noted the absence of a significant variation in the fecal IgA content comparing the experimental time points, i.e., pretreatment(T0), during (T15 and T30), and 30 days after *Lk* administration (T60).

Possible explanation of the results could be related to a poor immunomodulatory effect of *Lk* toward the canine GALT (gut associated lymphoid tissue) due to (a) poor *LK* viability in pharmaceuticals administered and consequent insufficient probiotic dose; (b) inadequate administration period; and (c) poor vitality in the gastrointestinal tract of the dog with lack of *Lk* probiotic activity in dogs.

Considering the first point, the differences found between the concentration declared by the company (*Lk* ≥ 25×10^9 AFU/capsule; ≥ 10^9 AFU/dose) and that found in our quality control analysis ($81 \pm 61 \times 10^7$ CFU/capsule; $3.2 \pm 2.4 \times 10^7$ CFU/dose) must be emphasized.

It must be stressed that the gap between what was declared by the probiotic company and what was highlighted by an independent analysis is not a rare event. In fact, analyzing the literature, numerous papers, both in the field of human and veterinary medicine, evidence this gap, with several-folds reduction of live probiotics concentration with respect to which reported by the companies (45–47).

Though not much is known about the minimal dose and/or frequency of probiotics required for the probiotic effect, it seems to be dose-dependent (48). For this reason, it cannot be excluded that the absence of changes observed in fecal microbiota and IgA during the trial with *Lk* can be attributed to an insufficient dosage, corresponding to ∼3% of the expected dose indicated for humans.

It is also true that an improvement in the enteric immune function in dogs was observed even after the administration of *Lactobacillus fermentum* at the dosage of 1×10^7 CFU/daily dose for 1 week, similar dose, and lower trial time, than the one actually used by us (18).

With respect to the treatment time, our study treatment is longer than employed in similar studies (15–17), with two intratreatment withdrawals in addition to pre- and post- treatment sampling, so this point can be excluded as a cause of poor response too.

Lastly, no previous studies have analyzed the *Lk* vitality in the canine gastrointestinal tract; therefore, it cannot be excluded that this may be the cause of the poor probiotic activity observed in our study.

Conversely, studies performed in human medicine have verified the gastrointestinal *Lk* vitality showing a high rate of adhesion of *Lk* to intestinal cells and strong resistance to gastric juice and intestinal bile salts (49).

Limitations of the study include the fact that dogs did not receive a uniform diet during the experiment, and the relatively low number of dogs included, further confounded by the exclusion of dog #6 from the analysis.

However, we believe that not artificially standardizing the dogs' diet might allow for results to be translated easier into real- life veterinary practice conditions and would showcase that *Lk* is able to impact the

microbiota independent of the diet given. We also believe that our methods of analysis will, to a certain degree, be able to counteract any dietary effect, as the dogs could serve as their own controls.

With respect to the sample size, the current study is similar to other feeding trials performing similar analyses (15–18).

In conclusion, our study was unable to demonstrate a significant change in microbiota composition or function in healthy dogs administered with *Lk* at a dose of $3.2 \pm 2.4 \times 10^7$ CFU/daily dose for 30 days. Further research will be necessary in order to assess the efficacy of a higher dose or of the combination of *Lk* with other potential probiotics.

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5 CONCLUSIONS

The purpose of this thesis was to review what was available in the literature regarding CIEs in dogs, including the latest updates. The frequency in clinical practice of dogs with acute and chronic gastrointestinal symptoms is high. It is therefore essential for the clinician to know how to properly set both the diagnostic and therapeutic protocol most suitable to manage CIE, bypassing useless and expensive steps. The development of increasingly less invasive diagnostic markers and the search for effective therapeutic approaches is constantly growing, with the aim of improving the well-being of these patients.

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REPORT ON PHD ACTIVITY

During the 2017-2020 doctoral period I carried out my research activity, focused on the field of gastroenterology and endoscopy of the dog, through the activity at the "Giuseppe Gentile" University Veterinary Hospital. I also actively participated in the practical teaching activity as part of the Veterinary Clinical Methodology course, Semeiotics and Medical Pathology of Companion Animals course and Companion Animals Internal Medicine. From 1 October 2019 to 17 January 2020 I carried out a period of externship at the “Rof Codina” Veterinary Hospital of the University of Santiago de Compostela.

Main projects carried out in the 3 years:

- Study on the incidence of bacteremia associated with endoscopic procedures in dogs: collection of blood samples to be subjected to blood culture with subsequent bacterial isolation in dogs undergoing different types of endoscopic procedures. Expansion of the series already started for the degree thesis.
- Study on the use of Lactobacillus kefiri as a probiotic in dogs: collection of feces in healthy dogs, which have taken Lactobacillus kefiri for 30 days, with evaluation of changes in the fecal microbiota and fecal IgA.
- Study on the FoxP3 factor: collaboration with the University of Pisa in the collection of cases to investigate the possibility of using the FoxP3 factor as a prognostic marker in the course of chronic enteropathies in dogs.
- Study on the use of water, as contrast media, during duodenoscopies in dogs: case studies to obtain a comparison in the quality of the endoscopic image obtained by insufflating water or air at the duodenal level in the dog and evaluation of anesthetic parameters during the procedure.
- Study on the use of coconut oil as an energy source in dogs with chronic enteropathy: collection of faecal samples in dogs with chronic enteropathy subjected to a home diet with a concentration of coconut oil known to evaluate any changes in the fecal microbiota level, Fecal IgA and composition of the fecal acidogram after the introduction of coconut oil in the diet. Comparison of the absorption capacity of fats supplied with the diet among animals with cobalamin values above 400ng / dL or below, considering cobalamin as a marker of intestinal absorption capacity in dogs. Project still in progress.

Pubblications

Bergamini, Ilaria; Linta, Nikolina; Gaspardo, Alba; Cunto, Marco; Peli, Angelo; Zambelli, Daniele; Pietra, Marco, Penile Foreign Bodies in Dogs: A Retrospective Study, *Acta Veterinaria-Beograd*, 2019, 69, pp. 450 – 460

Gaspardo, Alba; Zannoni, Augusta; Turroni, Silvia; Barone, Monica; Sabetti, Maria Chiara; Zanoni, Renato Giulio; Forni, Monica; Brigidi, Patrizia; Pietra, Marco, Influence of Lactobacillus kefiri on Intestinal Microbiota and Fecal IgA Content of Healthy Dogs, *Frontiers in Veterinary Science*, 2020, 7, pp. 1 – 11

Giorgia Galiazzo, Federico Costantino, Armando Foglia, Gabriele Bitelli, Noemi Romagnoli, Carlotta Lambertini, Chiara Francolini, Alba Gaspardo; Roberto Chiocchetti, Marco Pietra, Water Immersion vs. Gas Insufflation in Canine Duodenal Endoscopy: Is the Future Underwater?, *Polish Journal of Veterinary Science* 2020, DOI 10.24425/pjvs.2020.135804

Zannoni, Augusta; Pietra, Marco; Gaspardo, Alba; Accorsi, Pier Attilio; Barone, Monica; Turroni, Silvia; Laghi, Luca; Zhu, Chenglin; Brigidi, Patrizia; Forni, Monica, Non-invasive Assessment of Fecal Stress Biomarkers in Hunting Dogs During Exercise and at Rest, *Frontiers in Veterinary Science*, 2020, 7, pp. 1 – 16

Alba Gaspardo, Maria Chiara Sabetti, Renato Giulio Zanoni, Benedetto Morandi, Giorgia Galiazzo, Domenico Mion, Marco Pietra. (2020). Incidence of Bacteremia Consequent to Different Endoscopic Procedures in Dogs: A Preliminary Study. *Animals*, *10*(12), 2265.

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Partecipation at congress

- Flexible and rigid endoscopy of the digestive, respiratory, urinary and genital tract of small animals. Theoretical and practical course. Bologna (Italy) 2014, 2015, 2016, 2017, 2018, 2020.
- International Congress SCIVAC. Rimini (Italy) 2015, 2016 and 2018
- XII National UNISVET Congress, Milan (Italy) February 16-18, 2018.
- SIMIV Congress, Cremona (Italy) 21-22 April 2018. Scientific contribution →Clinical case: Pancytopenia in the course of sertolioma. SCIVAC International Congress, Rimini (Italy) 25-27 May 2018.
- 72 ° SISVet Congress, Turin (Italy) 20-22 June 2018. Scientific contribution →Short communication: Influence of Lactobacillus kefiri on gut IgA secretion in healthy dogs.
- SCIVAC National Gastroenterology Congress, Milan (Italy) 29-31 March 2019.