Effect of a novel dietary supplement (Relaxigen Pet dog®) on the fecal microbiome and stress-related behaviors in dogs

Simona Cannas[1*], Barbara Tonini[2], Benedetta Belà[3], Roberta Di Prinzio[3], Giulia Pignataro[3], Daniele Di Simone[4], Alessandro Gramenzi[3]

[1]Dipartimento di Medicina Veterinaria, Università degli Studi di Milano, Via Celoria 10, 20133, Milano, Italy

[2] via Vittorio Gassman, 15, 20128 Milano

[3] Veterinary Faculty, Località Piano D'Accio, 64100, Teramo, Italy

[4] Istituto di Fisiologia Clinica, CNR, Via G. Moruzzi 1, 56127, Pisa, Italy

***Corresponding author**

Dipartimento di Medicina Veterinaria, Università degli Studi di Milano, Via Celoria 10, 20133, Milano, Italy

Tel : +39 02 50318049; Fax: +39 02 50318030

E-mail address: simona.cannas@unimi.it; simona_cannas@hotmail.com (S. Cannas)

Abstract

Protracted stressful events determine behavioral and endocrine alterations and neuroinflammation (Carney & Gourkow, 2016; Fang et al., 2012). Furthermore, it was observed that anxiety and stress trigger functional gastrointestinal disorders, whereas the appearance of gastrointestinal symptoms can significantly increase anxiety and depression levels; also, the intestine and the brain are connected in what is today called the "gut-brain axis", a bi-directional communication of the nervous, endocrine and immune type (Zhu et al., 2017). This clinical trial aims to investigate the effects of a novel dietary supplement (Relaxigen Pet dog®) endowed with natural anti-inflammatory compounds (CLA, Krill), pre/probiotics, 5-HTP and L-theanine on stress-related behavior and deepen on the connection between these stress-related behaviors and fecal microbiome in dogs. Forty dogs, ranging in ages from 1 to 10 years, took part in this double-blind placebo clinical trial: 30 dogs with signs of stress and anxiety (randomly divided in "therapy" and "placebo" group) and 10 control dogs. The therapy sample group (20 dogs balanced for sex) was administered with Relaxigen pet dog® and the placebo one (10 dogs balanced for sex) with a placebo for 60 days. A basic history questionnaire focused on all aspects of dog's behavior, management, and health issue was collected at time 0, 30 days and 60 days for each dog, including several variables on signs of stress and anxiety using a 0-5 Likert scale. For each dog of the three groups, a fecal sampling was collected at time 0 and for the "therapy" and "placebo" group also at 30 and 60 days so to extract the DNA for microbiological analysis and determine the leading bacterial group using a Quantitative Real-Time PCR. The Anova showed an influence of the treatment in conjunction with the time passed, and during the same period, the group that received the treatment had a probability of improving greater than 10% ($p \leq 0.05$).

Moreover, the results of this study highlighted a different structure of the intestinal microbiota between healthy and dogs with stress-related behaviors. Supplementation with the Relaxigen product has determined some changes in the concentration of bacterial groups taken into account. Further works are needed to investigate whether the conclusion drawn from this population can be generalized to dogs with different problem behavior and to deepen the relationship between these disorders and the gut-brain axis.

Keywords*: gut-brain axis, fecal microbiome, stress-related behavior, dogs*

Introduction

The animal organism has an adaptive response to real or supposed dangers that include two different mechanisms to alleviate a state of stress in adverse situations that threaten homeostasis. These responses consist of developing behavioral changes aiming to annul the effect of the threat and physiological changes that are necessary to restore and maintain internal homeostasis (Casey, 2002).

When an animal is unable to escape the stressor through an appropriate behavioral response, the stress response becomes chronic, and that leads to adverse effects on the physical and emotional state of the individual (Casey, 2002).

When the stress response is prolonged or when the stressor persists, the emotional response to ensure that the animal escapes the situation also continues. In dogs, chronic stress underlies a wide range of behavioral problems such as anxiety, fear, and aggression (Carney and Gourkow, 2016; Casey, 2002).

These common behavioral diseases compromise biological functions inducing altered well-being and a poor quality of life (Cahill and McGaugh, 1998, 1995; Carney and Gourkow, 2016; Casey, 2002). In protracted stressful events, the individual becomes unable to exploit an effective behavioral mechanism for reducing his physiological response whilst the cortisol produced in excess turns out to be negative at different levels on the individual's body: hypertension, diabetes, infertility, growth inhibition, loss of libido, reduction of the level of attention, alteration of memory, inhibition of inflammatory responses and alteration of immune function (Cahill and McGaugh, 1998, 1995; Casey, 2002).

In particular, protracted stressful events seem to determine behavioral and endocrine alterations and neuroinflammation (Carney and Gourkow, 2016; Fang et al., 2012).

Inflammation that acts as a protective function in controlling infections and promoting tissue repair is usually self-limited. However, a prolonged or repeated stimulus delays the release of pro-inflammatory mediators and neurotoxins that worse tissue damage and negatively impact disease outcome leading to an anxiety-like behavior state (Wohleb et al., 2015). Although the biological mechanisms connecting neuroinflammation to mental health complications are not well-understood, several studies in humans underline how inflammation and altered immune signaling significantly contribute to the etiology of many psychiatric symptoms and disorders (Evans et al., 2005), depression (Walker et al., 2014), sickness behavior (Biesmans et al., 2013) anxiety (Pace and Heim, 2011) and cognitive decline also in dogs (Frank-Cannon et al., 2009; Smolek et al., 2016).

Furthermore, anxiety and stress appeared to trigger functional gastrointestinal disorders, as well as the appearance of gastrointestinal symptoms, they can significantly increase anxiety and depression

levels; the intestine and the brain are connected in what is today called the "gut-brain axis", a bi-directional communication of the nervous, endocrine and immune type (Zhu et al., 2017). The gut is considered the fulcrum of the body's health both for the intestinal barrier, that represents the widest interface of the entire organism with the external world, then for the intestinal microbiota. The intestinal microbiota, a microbial ecosystem made up of billions of microorganisms, is able to influence physiological, behavioral and cognitive functions of the brain (Jenkins et al., 2016; Mayer et al., 2014; Peter and Smith, 2015; Schmidt, 2015), conversely, intestinal dysbiosis can activate the immune system, leading to an excessive release of pro-inflammatory cytokines that negatively affect brain function. Preliminary reports suggested that the administration of beneficial microbes could be helpful in cases of depression (Norman, 1909) and dietary alterations, especially macronutrients, were part of mainstream discussions concerning ways to manipulate intestinal microbiota for health (Editors of Jama, 1919); for example, manipulating dietary protein and carbohydrate in animals could induce changes in behavior with associated changes in the microbiota. The behavioral changes were attributed to a combination of both direct macronutrient influences on mood and the indirect ability of foods to shift the production of mood-altering microbial byproducts (Herter and Kendall, 1910).

The aim of this clinical trial was to investigate the effects of a novel dietary supplement (Relaxigen Pet dog®) endowed with natural anti-inflammatory compounds (CLA, Krill), pre/probiotics (*Lactobacillus reuteri inactivated*, butyric acid, FOS), 5-hydroxytryptophan and L-theanine, as an adjuvant in the treatment in stress-related behavior in dogs. One hypothesis of current work is that sustained neuroinflammation may be one of the factors triggering stress-induced anxiety disorders and a supplement contrasting inflammation may be useful for the treatment in stress-related behavior.

Moreover, this study wants to deepen the connection between stress-related behavior and fecal microbiome in dogs.

Materials and Methods

Study design

The study was a double-blind placebo clinical trial.

Subject

40 dogs, ranging in ages from 1 to 10 years, took part in this study: 30 dogs with signs of stress and anxiety and 10 dogs without these signs (hereafter named "control group"). Dogs' evaluation was done by a veterinary behaviorist (always the same person).

The 30 dogs with signs of stress and anxiety were randomly divided into two groups: a therapy group and the placebo group.

The "therapy group" consisted of 20 dogs balanced for sex (10 dogs < 20kg and 10 dogs>20kg), administered with Relaxigen pet dog®.

The "placebo group" consisted of 10 dogs balanced for sex (5 dogs < 20kg and 5 dogs>20kg) administered with a placebo.

All the dogs belonging to the therapy and placebo group were subjected to a behavioral modification prescribed by the same veterinary behaviorist that evaluated the dog during the enrolment.

Between winter and summer 2018, subjects were recruited when they met all the inclusion criteria, whereas, they were excluded from participation when they met any of the exclusion criteria (Table 1).

Table 1. Inclusion and exclusion criteria for subject dogs

INCLUSION	EXCLUSION
Older than 1 year, younger than 10 years (1 through 9 inclusive)	Chronic medical problems noted by medical records, physical examination, or laboratory analysis
Either sex, intact or neutered	Treatment with psychotropic, anxiolytic, or sedative medications
Any breed, known or unknown	Pregnant or lactating
Client owned for at least the past 12 months	

Experimental phase

Each dog of the therapy and placebo group was administered for per 60 days with the Relaxigen pet dog® or with the placebo according to the experimental group. Aspect and modality of administration were the same for the product and the placebo.

Relaxigen pet dog® and placebo composition are represented in table 2.

Krill is characterized by high levels of omega-3 EPA and DHA in phospholipid form that allows their better incorporation in the brain. The EPA and DHA perform various neuroprotective actions, as precursors of molecules able to favor the functional restoration of neurons damaged by chronic stress and carry out the anti-depressive activity (Bazinet and Layé, 2014; Choi et al., 2017; Kiecolt-Glaser et al., 2011; Rapaport et al., 2016).

Conjugated linoleic acid (CLA) has numerous functional properties including anti-inflammatory activity at the level of the central nervous system; in fact, the CLA passes the blood-brain barrier and acts through nuclear receptors, triggering the production of molecules with anti-inflammatory activity, protecting the nerve cells from chronic stress damage with stabilization of the mood by restoring the dopaminergic system and allowing a physiological response adequate to the different stressful insults (Belury, 2002; Fa et al., 2005).

The simultaneous presence of tyndallized probiotics (L. reuteri *), prebiotics (FOS) and post-biotics

(butyric acid) grant a complete and effective control against intestinal dysbiosis conditions so to allow a normal hypothalamic-pituitary-adrenal axis response to the stress.

The positive modulation of the intestinal microbiota allows to act on both the endocrine and immune cells of the intestinal wall and on the nerve endings thus setting up the enteric nervous system associated with the nerve structures of the spinal cord and conveying signals deriving from the gut to reach the brain. This modulation induce the reduction of anxiety levels and change the activation of the brain regions that control emotions.

Intermediate metabolite of the essential amino acid L-tryptophan (LT) in serotonin biosynthesis; while the LT, as well as the serotonin, is also a precursor of niacin and some proteins, 5-HTP is used exclusively for the synthesis of serotonin in an extremely efficient, as it bypasses the conversion of LT into 5-HTP by the enzyme tryptophan hydroxylase, which is the limiting factor in the synthesis of serotonin. 5-HTP easily crosses the blood-brain barrier by effectively increasing the synthesis of serotonin in the central nervous system where it is involved in the regulation of sleep, depression, anxiety, aggression, apathy, thermoregulation, sexual behavior, and sensation of pain (Birdsall, 1998; Turner et al., 2006).

L-TEANINA is a derivative of glutamic acid, one of the fundamental amino acids for a good functioning of the central nervous system because it is a precursor of the biosynthesis of gamma-aminobutyric acid (GABA), an important neurotransmitter with a central inhibitory action. Scientific evidence shows that L-theanine implements the levels of some chemical mediators, such as dopamine and serotonin, which are involved in a more or less direct way in the control and regulation of behavior, of cognitive processes and above all of the emotions (Camfield et al., 2014; Lu et al., 2004). The L-theanine, unlike other active constituents of plant origin with anxiolytic and relaxing action, does not cause drowsiness, therefore offers the possibility of having relaxation without sedation.

Table 2. Relaxigen pet dog® and placebo composition (for 1 g).

RELAXIGEN PET DOG®	mg	PLACEBO	mg
5-hydroxy tryptophan	50	5-hydroxy tryptophan	--
L-theanine	50	L-theanine	--
Conjugated linoleic acid	225	Conjugated linoleic acid	--
Krill oil	225	Krill oil	--
L. reuteri NBF1*	100	L. NBF1*	--
Butyric acid	100	Butyric acid	--
Fructo-oligosaccharides	50	Fructo-oligosaccharides	--
Tocopherol acetate	2	Tocopherol acetate	2
Meat flavour	50	Meat flavour	50
Anhydrous silica	80	Anhydrous silica	80
Sunflower oil	as much as 1 g	Sunflower oil	as much as 1 g

Questionnaire

A basic history questionnaire focused on all aspects of the dog's behavior, management, and health issue was collected of the therapy and placebo group. Several background questions, regarding early life experiences such as socialization and the puppy period, were included, as well as more general questions concerning daily routines and diet, with food types and extra nutrients.

Several variables of the dog's behavior and signs of stress and anxiety were collected using a 0-5 Likert scale (0=never-5= very frequently) at time 0, 30 days, and 60 days.

A global assessment, based on the owner's perception of the animal's improvement, was recorded at 30 and 60 days.

These scores were based on subjective owner assessment. Owners were blind regarding the group in which his dog was included.

Fecal sampling

For each dog of the three groups, a fecal sampling was collected at time 0 and for each dog of the "therapy" and "placebo" group also at 30 and 60 days.

DNA extraction from fecal samples for microbiological analysis

DNA extraction from fecal samples was performed using a modified method with benzyl chloride (Zhu et al., 1993). Briefly, 250 µl of extraction buffer (Tris-HCl 100 mM, EDTA, pH 9), 50 µl of SDS 10% and 150 µl of benzyl chloride were added to 1 g of fecal samples. Eppendorf tubes were homogenized by vortex and incubated at 50°C for 30 minutes using the thermomixer; 150 µl of sodium acetate 3 M was then added and samples were kept on ice for 5 minutes. After centrifugation at 13000 rpm at 4°C for 12 minutes, the supernatant was harvested, and the DNA precipitated with isopropanol; after wash with ethanol 70%, the precipitated DNA was resuspended in 50 µl of TE buffer and stored at -20°C.

Quantitative Real-Time PCR determination of the main bacterial group

The quantitative determination of the main bacterial groups present at the intestinal level was performed using a quantitative PCR (qPCR) procedure in real-time, as reported by Nasuti *et al*. (2016). The bacterial groups analyzed were the following: *Lactobacillus* spp., *Bifidobacterium* spp., *Enterobacteriaceae*, *Clostridium coccoides- Eubacterium rectale* group, *Staphylococcus* spp., *Bacteroides- Prevotella- Porphyromonas* spp. Table 3 shows the primers used in Real-Time PCR reactions with the reference strains used for the preparation of standard curves. All Real-Time PCR determinations/reactions have been performed in duplicate.

Table 3. Sequences of primers used to amplify reference bacterial strains.

Bacterial reference strain	Primer sequences (5'-3')	References

Bacteriodes fragilis DSM2151	F: GGTGTCGGCTTAAGTGCCAT R: CGGAYGTAAGGGCCGTGC	Rintilla *et al*. 2004
Staphylococcus aureus ATCC 29213	F: GCGATTGATGGTGATACGGTT R: AGCCAAGCCTTGACGAACTAAAGC	Fang & Hedin 2003
Blautia producta DSM 2950	F: CGGTACCTGACTAAGAAGC R: AGTTTYATTCTTGCGAACG	Rintilla *et al*. 2004
Lactobacillus acidophilus ATCC 314	F: TGGAAACAGRTGCTAATACCG R: GTCCATTGTGGAAGATTCCC	Byun *et al*. 2004
Bifidobacterium longum DSM 20219	F: GGGTGGTAATGCCGGATG R: TAAGCGATGGACTTTCACACC	Langendijk *et al*. 1995
Escherichia coli ATCC 25288	F: CATTGACGTTACCCGCAGAAGAAGC R: CTCTACGAGACTCAAGCTTGC	Bartosch *et al*. 2004

Real-time PCR amplification was performed using an iCycler iQ Real-Time Detection System (Stratagene) associated with an MXP Software. Reaction mixtures contained: 9.8 µl of Maxima SYBR Green qPCR Master Mix (Thermo scientific), 9 µl sterile distilled water and 0.4 µl of each primer (Forward and Reverse, 500 ng/µl). Subsequently, 1 ml of DNA (or DNA-free water for a negative control) was added to the reaction mixture. The amplification was carried out at the initial temperature of 95°C for 2 minutes to activate the Universal SYBR probe and then following the amplification protocols shown in Table 4.

Table 4. Specific amplification protocols for each target bacterial group.

Target	Primer sequence	Reference strain	Denaturation	Annealing	Extension	Cycles
Bacteroides-Prevotella-Porphyromonas spp.	F: GGTGTCGGCTTAAGTGCCAT R: CGGAYGTAAGGGCCGTGC	*Bacteroides fragilis* DSM 2151	95°C 15 sec	58°C 20 sec	72°C 30 sec	35
Staphylococcus spp.	F: GCGATTGATGGTGATACGGTT R: AGCCAAGCCTTGACGAACTAAAGC	*Staphylococcus aureus* ATCC 29213	95°C 15 sec	49°C 20 sec	72°C 30 sec	35
Clostridium coccoides-Eubacterium rectale group	F: CGGTACCTGACTAAGAAGC R: AGTTTYATTCTTGCGAACG	*Blautia producta* DSM 2950	95°C 15 sec	55°C 20 sec	72°C 30 sec	35
Lactobacillus spp.	F: TGGAAACAGRTGCTAATCCG R: GTCCATTGTGGAAGATTCCC	*Lactobacillus acidophilus* ATCC 314	95°C 15 sec	47°C 1 min	72°C 1 min	40
Bifidobacterium spp.	F: GGGTGGTAATGCCGGATG R: TAAGCGATGGACTTTCACACC	*Bifidobacterium longum* DSM 20219	95°C 30 sec	59°C 30 sec	72°C 45 sec	35
Enterobacteriaceae	F: CATTGACGTTACCCGCAGAAGAAGC R: CTCTACGAGACTCAAGCTTGC	*Escherichia coli* ATCC 25288	95°C 30 sec	55°C 30 sec	72°C 45 sec	35

Statistical analysis of bacterial enumeration

The significant differences between the mean values of the count of microorganisms in the different groups of analyzed samples were determined by the Tukey Test after the analysis of the one-way variance. A value of P equal or less than 0.05 was considered significant. The statistical analysis was performed using the GRAPHPAD PRISM 5.1® program (GraphPad Software, San Diego, CA, USA).

Data Analysis

Answers to the questionnaires were scored and analyzed using IBM SPSS Statistics 25 (SPSS Inc., Chicago). A descriptive statistical analysis was performed. The proportion test was used to evaluate differences between the two groups of dogs at time zero and differences within the therapy group between time 0 and time 2 (after 60 days). To evaluate the treatment effect and the influence of the time on our model, a Repeated Measures Anova was performed.

Results

Questionnaire

Our sample was composed by 15 males (10 intact males and 5 neutered) and 15 females (3 intact females and 12 spayed), ranging in age from 1 to 12 years (mean 3,27 ± 2,73 years). 33% of dogs were mixed breeds, and 67% was a pure breed. More information about breeds is specified in Table 5. 36,7 % were large and small-sized dogs, and 26,7% were medium. The most of dogs were fed with a commercial diet, 16.7 % with home-based diet and just one dog with BARF and one utilized a mixed diet (commercial and home-based). All of the owners utilized snacks for their dogs. Most of the subjects (40%) were adopted directly from breeders, the others came from a shelter or were strays (36,6%), were transferred from another person (16,6%), (13%) or and the remaining dogs (6,7%). The most of dogs (70,1%) were adopted after 75 days of age, 26.6% between 51 and 75 days of age, and just one subject before 51 days. 76,7% of dogs lived in an apartment, and 23,4% lived in a house. Based on owners answers, 66,7% of dogs showed aggressive behaviors; in particular, 63,3% showed growling and 53,3% bite attempts. 63,3 % of dogs appeared nervous (had difficulty relaxing), 70% always alert, and 53,3% tend to hide and isolate itself. The majority of our sample (76,7%) was fearful of noises. 56,7% of dogs showed frequent vomiting episodes and 73,3% frequent diarrhea. No differences were found between the therapy group and the placebo one at time zero ($p \leq 0.05$). In the therapy group, all the previous behaviors showed a significant changing (while no changes were seen in the control group): the percentage of dogs that didn't show aggressive behaviors increased from 30% to 75% ($p \leq 0.05$). A similar trend was seen for growling and bite attempts: the percentage of dogs that didn't show these behaviors increased respectively from 35% to 75% and from 40% to 90% ($p \leq 0.05$). The percentage of dogs that didn't

appear nervous (nor showing a difficulty at relaxing) increased from 25% to 60%, that of those who weren't always alert increased from 15% to 45% and the percentage of those who didn't tend to hide and isolate themselves increased from 30 to 60% ($p \leq 0.05$). The percentage of dogs that was fearful of noises and that reported the point 5 in the Likert scale (indicating a very frequent presence of the behavior) decreased from 45% to 10% ($p \leq 0.05$). Dogs that didn't show frequent vomiting episodes and frequent diarrhea increased from respectively from 35% to 75% and from 10% to 50% ($p \leq 0.05$).

Based on owners' answers, 95% of dogs of the therapy group showed an improvement, while just 50% of dogs of the control group showed improvement ($p \leq 0.05$).

The Repeated Measures Anova showed an influence of the treatment in conjunction with the length of treatment time. Under comparable treatment period, the group that received the nutrition supplement had a probability to improve greater than 10% ($p \leq 0.05$).

Table 5. Dog Breed distribution.

BREED	FREQUENCY	%
Border collie	1	3,3
Bracco Italiano Dog	1	3,3
French Bulldog	1	3,3
Cavalier King Charles	1	3,3
Chihuahua	4	13,3
Papillon	1	3,3
J R Terrier	1	3,3
Golden Retriever	1	3,3
Labrador Retriever	2	6,7
Malinois	1	3,3
Maltese dog	1	3,3
German Shepherd	1	3,3
Shih-Tzu	3	10
Mixed breed	11	36,6

Bacterial enumeration

The results of the quantification of the bacterial groups in the analyzed fecal samples are given in Figures 1, 2, 3, 4, 5, and 6. The composition of the intestinal microbiota of dogs in the study showed in all groups at T0 a prevalence of the Clostridium coccoides- Eubacterium rectale group, followed by the Bacteroides- Prevotella- Porphyromonas spp., by the analysis data of canine fecal microbiome analyzed by other authors (Suchodolski et al., 2008). The group Bacteroides- Prevotella- Porphyromonas spp. suffers a significant decrease in the Therapy group at T2 compared

to the Control (Fig. 1). Anxious dogs, both Treated and Placebo, showed a concentration of lactobacilli (Fig. 5), bifidobacteria (Fig. 2) and Enterobacteriaceae (Fig. 4) significantly higher than the Control. In anxious dogs undergoing treatment with Relaxigen, there is a decrease of bifidobacteria both at T1 and T2 (Fig. 2) and a statistically significant decrease of lactobacilli both in relation to the Control and the Placebo group (Fig. 5). In a study on the effects of stress on the canine microbiome (Venable et al., 2016), an increase in bifidobacteria was recorded in dogs subjected to flight stress. The Clostridium coccoides- Eubacterium rectale group undergoes a statistically significant decrease in the Therapy group at T2 concerning both Control and Placebo group (Fig. 3). Enterobacteriaceae significantly decrease in the Therapy group at T2 compared to Placebo. Also a high concentration of Enterobacteriaceae in the intestinal microbiome of dogs, is associated with particular pathological states (Honneffer et al., 2014) and thus the decrease observed in the Therapy group at T1 and T2, not registered instead in the Placebo group, is certainly a positive result and to be subjected to further investigation. Staphylococcus spp. showed no variation in the three examined groups.

Discussion

This study aimed to investigate the effects of a novel dietary supplement (Relaxigen Pet dog®) as an adjuvant in the treatment in stress-related behavior in dogs and deepen in the connection between stress-related behavior and microbiota in dogs.

Stress responses to external stimuli become problematic when an individual animal is unable to control the situation or to escape from the stressor through an appropriate behavioral response, and the stress response becomes prolonged or chronic (Casey, 2002; Notari, 2009; Weiss, 1972).

When the stressor is chronic, the animals will also exhibit inappropriate or excessive behavioral responses to lower the level of a prolonged stress response (Dantzer and Mormede, 1981).

In our work, no difference was found at time 0 between the two groups (therapy and placebo), suggesting that the two groups were homogeneous: essential data to evaluate the effects of the treatment. The dogs belonging to the therapy group showed a lowering in aggressive behavior, nervousness (difficulty in relaxing), alertness, hiding and isolating, and fearful behaviors. Moreover, in these dogs decreased vomiting episodes and diarrhea. Behavioral responses of dogs to situations they perceive as stressful are avoidance, aggressiveness, panting, salivation, pacing, hyperactivity, hypervigilance, elimination, gastrointestinal disorder, hiding, digging, flattened ear, anorexia, attention-seeking, lip-licking, frequent swallowing, low tail position (Beerda et al., 1998; Notari, 2009; Overall, 2013; Rooney et al., 2007).

Stress events trigger neuronal microtraumatism and neuroinflammatory activation in the brain (Wager-Smith and Markou, 2011). In the brain, inflammatory mediators are mainly produced by

endothelial and glial cells, including astrocytes and microglia (Jha et al., 2016). Social stress may activate microglial cells through the activation of glucocorticoid and mineralocorticoid (Sierra et al., 2008) and β-adrenergic receptors (Calcia et al., 2016).

The activation of microglia decreased brain-derived neurotrophic factor and release higher levels of interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) that reduce the availability of serotonin, dopamine, and noradrenaline (Miller and Raison, 2017).

Due to potentiation of the brain's main inhibitory transmitter GABA, L-theanine might act as a mild anxiolytic (Junej et al., 1999) and 5-Hydroxytryptophan (5-HTP) is an amino acid precursor used in the formation of serotonin (Lee, 2018), so their use in this dietary supplement could help in case of stress-related behaviour. Moreover, the presence of the EPA, DHA, and Conjugated linoleic acid (CLA) could help to perform various neuroprotective and anti-inflammatory actions (Bazinet and Layé, 2014; Belury, 2002; Choi et al., 2017; Fa et al., 2005; Kiecolt-Glaser et al., 2011; Rapaport et al., 2016).

The results of this study highlighted a different structure of the intestinal microbiota between healthy and anxious dogs. Supplementation with the Relaxigen product has determined some changes in the concentration of bacterial groups taken into account.

In the gut microbiota-brain axis, because gut microbiota can be used as an independent variable and changed intentionally, more emphases are placed on the role of microbes in gut microbiota-brain axis (Omran and Aziz, 2014). The gut can interact with the brain through two neuroanatomical pathways. The one is mutual information exchange directly between the gut and brain by the autonomic nervous system (ANS) and vagus nerve (VN) in the spinal cord; another one is a bidirectional communication between gut and brain through the bi-communication between enteric nervous system (ENS) in the gut and ANS and VN within the spinal cord. The anatomical neural pathways for controlling gut functions form a hierarchic "four level" integrative organization (Foster and McVey Neufeld, 2013; Mulak and Bonaz, 2015): the first level is the ENS, including myenteric ganglia, submucous ganglion, and gut glial cells (Anlauf et al., 2003; Schemann and Neunlist, 2004), the second level is prevertebral ganglia regulating peripheral visceral reflex responses (Szurszewski, 2003), the third level is the ANS in the spinal cord (from T5 to L2 sympathetic nerve and S2 to S4 parasympathetic nervous system) and brain stem nucleus tractus solitarius and dorsal motor nucleus of VN, which receive and give the origin of afferent and efferent fiber of VN, respectively. The most important effect of the dorsal motor nucleus of VN is prominent in the upper gastrointestinal tract, and the cholinergic neurons on myenteron of upper gastrointestinal tract regulate vagal excitability effect (Chang et al., 2003), and the fourth level is the higher brain centers. Information from cortex and subcortical centers including basal ganglia

and funnels down to peculiar brainstem nuclei; brainstem nuclei control many gut functions. The afferent fiber of VN stops at the brain stem nucleus tractus solitarius, which then gives fiber upward and arrives at the thalamus, lobus limbicus, and insular cortex through the parabrachial nucleus. Spinal afferent fiber goes upward within the spinothalamic tract and spinal tract to the thalamus (spinothalamic tract) and gracile nucleus and cuneate nucleus of the medulla oblongata (spinal tract), respectively, then project fiber to thalamus through lemniscus medialis. Fiber is given from the thalamus and projected to the primary sensorimotor areas and insular cortex. Damages and abnormalities at the levels mentioned above can influence the regulation of intestinal function, including local intestinal reflexes, and external neural control (Mulak and Bonaz, 2015). Direct neural communication between gut microbiota and the brain is mainly realized through VN, i.e., bacteria stimulates afferent neurons of ENS, and the vagal signal from the gut can stimulate the antinflammatory response, preventing against pyosepticemia caused by microorganisms.

Further research showed that many effects of gut microbiota or potential probiotics on brain functions were independent on vagal activation (Forsythe et al., 2014) and bacteria settled in the gut played a critically important role in individual's postnatal development and the maturation of the immune system, the endocrine system, and the nervous system (Borre et al., 2014). However, generally, the vagus nerve is a confirmed conduit for promoting anxious behavior following induction of intestinal inflammation and anxiolysis following administration of a probiotic (Bercik et al., 2011). Probiotic supplementation to healthy animals reduces anxiety and depression-like behavior in various models of stress-induction (Bravo et al., 2011).

Critically, behavioral changes are associated with changes in the expression of gamma-aminobutyric acid (GABA) receptors in areas of the brain governing emotion. Again, the vagus nerve appears to be the channel between beneficial microbes, behavior, and brain chemistry (Bravo et al., 2011). Beneficial microbes are also known to suppress histamine signaling in allergy models (Dev et al., 2008). Additionally, also the development of gut immune system seems to depend on gut microbiota (Furusawa et al., 2013); bacteria communicate with the host through a variety of ways, and the "receptors TLRs" of host cell plays a key role in the communication between bacteria and host. There are different kinds of TLRs in the innate immune system, which have been identified as pattern recognition receptors (Takeuchi and Akira, 2010). These receptors are a part of the innate immune system, which is the first step to produce cytokine response and is also widely distributed on neurons (McKernan et al., 2011). Hence, neurons also respond to bacterial and viral components. Intestinal epithelial cells can transport microbial composition or metabolites into the inner environment, and the nervous system also interacts with these bacterial and viral components (O'Brien et al., 2004). The balance of gut microbiota may change the regulation of inflammatory

response, and this mechanism may also get involved in the regulation of emotion and behavior (Foster and McVey Neufeld, 2013; Levkovich et al., 2013). These findings suggest that modulating the composition of the intestinal microbiota may induce an improvement even in dog behavior.

In conclusion, this study provided evidence that Relaxigen Pet dog® may be used as support in alleviating stress in dogs combined with behavioral therapy. For the therapy group, dogs' owners perceived a general improvement of their dogs, and the statistical analysis showed an influence of the treatment in conjunction with the time passed. In literature, there is increasing evidence that the manipulation of the intestinal microbiota can specifically affect anxious behavior (Collins et al., 2013; Neufeld et al., 2011). Shaping the intestinal microbiota through integration with specific functional ingredients could be a way to optimize the overall health of the animals, and, consequently, improve their well-being.

Further works are needed to investigate whether the conclusion drawn from this population can be generalized to dogs with different problem behavior and to deepen on the relation between these disorders and the gut-brain axis. More thorough studies are needed, which should be addressed to the identification of factors such as race, age, dietary changes, which could compose a variability of the dog's gastrointestinal microbiome.

Acknowledgments

The Authors thank dr. Carlo Maria Colombo e dr. Giuseppe Pappini of NBF Lanes (Milan), to have supported the study.

Ethics approval

The research protocol, under article 2, paragraph 1, letter b falls within the cases excluded from the application of the legislative decree 4 March 2014, n. 26.

Authorship statement

The idea for the paper was conceived by Alessandro Gramenzi, Barbara Tonini and Simona Cannas.

The experiments were designed by Barbara Tonini, Simona Cannas, and Alessandro Gramenzi.

The experiments were performed by Simona Cannas e Barbara Tonini.

The data were analyzed by Daniele Di Simone, Benedetta Belà, Roberta Di Prinzio, Giulia Pignataro and Simona Cannas.

The paper was written by Simona Cannas, Barbara Tonini and Alessandro Gramenzi.

Conflict of interest statement

The authors of this paper do not have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

References

Anlauf, M., Schäfer, M.K.H., Eiden, L., Weihe, E., 2003. Chemical coding of the human gastrointestinal nervous system: Cholinergic, VIPergic, and catecholaminergic phenotypes. J. Comp. Neurol. 459, 90–111.

Bazinet, R.P., Layé, S., 2014. Polyunsaturated fatty acids and their metabolites in brain function and disease. Nat. Rev. Neurosci. 15, 771–785.

Beerda, B., Schilder, M.B.H., Van Hooff, J.A.R.A.M., De Vries, H.W., Mol, J.A., 1998. Behavioural, saliva cortisol and heart rate responses to different types of stimuli in dogs. Appl. Anim. Behav. Sci. 58, 365–381.

Belury, M.A., 2002. Dietary conjugated linoleic acid in health: Physiological effects and mechanisms of action. Annu. Rev. Nutr. 22, 505–531.

Bercik, P., Park, A.J., Sinclair, D., Khoshdel, A., Lu, J., Huang, X., Deng, P.A., 2011. The anxiolytic effect of Bifidobacterium longum NCC3001. Neurogastroenterol. Motil. 23, 1132–1139.

Biesmans, S., Matthews, L.J.R., Nuydens, R., Bouwknecht, J.A., De Haes, P., Meert, T.F., Acton, P.D., Davoodi, N., Kuijlaars, J., Langlois, X., Hellings, N., Ver Donck, L., 2013. Systemic Immune Activation Leads to Neuroinflammation and Sickness Behavior in Mice. Mediators Inflamm. 2013, 1–14.

Birdsall, T.C., 1998. 5-Hydroxytryptophan: A Clinically-Effective Serotonin Precursor. Altern. Med. Rev. 3, 271–280.

Borre, Y.E., O'Keeffe, G.W., Clarke, G., Stanton, C., Dinan, T.G., Cryan, J.F., 2014. Microbiota and neurodevelopmental windows: implications for brain disorders. Trends Mol. Med. 20, 509–518.

Bravo, J.A., Forsythe, P., Chew, M. V., Escaravage, E., Savignac, H.M., Dinan, T.G., Bienenstock, J., Cryan, J.F., 2011. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc. Natl. Acad. Sci. 108, 16050–16055.

Cahill, L., McGaugh, J.L., 1995. Recall of Emotionally Arousing Stories (Updated). Conscious. Cogn. 4, 410–421.

Cahill, L., McGaugh, J.L., 1998. Mechanisms of emotional arousal and lasting declarative memory. Trends Neurosci. 21, 294–299.

Calcia, M.A., Bonsall, D.R., Bloomfield, P.S., Selvaraj, S., Barichello, T., Howes, O.D., 2016. Stress and neuroinflammation: A systematic review of the effects of stress on microglia and the implications for mental illness. Psychopharmacology (Berl). 233, 1637–1650.

457 Camfield, D.A., Stough, C., Farrimond, J., Scholey, A.B., 2014. Acute effects of tea constituents L-
458 theanine, caffeine, and epigallocatechin gallate on cognitive function and mood: A systematic
459 review and meta-analysis. Nutr. Rev. 72, 507–522.
460 Carney, H., Gourkow, N., 2016. Impact of stress and distress on cat behavior and body language.
461 In: Ellis, S., Sparkes, A. (Eds.), ISFM Guide to Feline Stress and Health. Managing Negative
462 Emotions to Improve Feline Health and Wellbeing. International Cat Care, Tisbury, Wiltshire,
463 pp. 31–39.
464 Casey, R., 2002. Fear and stress in companion animals. In: Horwitz, D., Mills, D., Heath, S. (Eds.),
465 BSAVA Manual of Canine and Feline Behavioural Medicine. British Small Animal Veterinary
466 Association, Gloucester, pp. 144–153.
467 Chang, H.Y., Mashimo, H., Goyal, R.K., 2003. IV. Current concepts of vagal efferent projections to
468 the gut. Am. J. Physiol. Liver Physiol. 284, G357–G366.
469 Choi, J.Y., Jang, J.S., Son, D.J., Im, H.S., Kim, J.Y., Park, J.E., Choi, W.R., Han, S.B., Hong, J.T.,
470 2017. Antarctic krill oil diet protects against lipopolysaccharide-induced oxidative stress,
471 neuroinflammation and cognitive impairment. Int. J. Mol. Sci. 18.
472 Collins, S.M., Kassam, Z., Bercik, P., 2013. The adoptive transfer of behavioral phenotype via the
473 intestinal microbiota: Experimental evidence and clinical implications. Curr. Opin. Microbiol.
474 16, 240–245.
475 Dantzer, R., Mormede, P., 1981. Pituitary adrenal consequences of adjunc- tive behaviours in pigs.
476 Horm. Behav. 15, 386–395.
477 Dev, S., Mizuguchi, H., Das, A.K., Matsushita, C., Maeyama, K., Umehara, H., Ohtoshi, T.,
478 Kojima, J., Nishida, K., Takahashi, K., Fukui, H., 2008. Suppression of Histamine Signaling
479 by Probiotic Lac-B: a Possible Mechanism of Its Anti-allergic Effect. J. Pharmacol. Sci. 107,
480 159–166.
481 Editors of Jama, 1919. Food and the control of bacteria in the intestine. J. Am. Med. Assoc. 72,
482 1370.
483 Evans, D.L., Charney, D.S., Lewis, L., Golden, R.N., Gorman, J.M., Krishnan, K.R.R., Nemeroff,
484 C.B., Bremner, J.D., Carney, R.M., Coyne, J.C., Delong, M.R., Frasure-Smith, N., Glassman,
485 A.H., Gold, P.W., Grant, I., Gwyther, L., Ironson, G., Johnson, R.L., Kanner, A.M., Katon,
486 W.J., Kaufmann, P.G., Keefe, F.J., Ketter, T., Laughren, T.P., Leserman, J., Lyketsos, C.G.,
487 McDonald, W.M., McEwen, B.S., Miller, A.H., Musselman, D., O'Connor, C., Petitto, J.M.,
488 Pollock, B.G., Robinson, R.G., Roose, S.P., Rowland, J., Sheline, Y., Sheps, D.S., Simon, G.,
489 Spiegel, D., Stunkard, A., Sunderland, T., Tibbits, P., Valvo, W.J., 2005. Mood disorders in
490 the medically ill: Scientific review and recommendations. Biol. Psychiatry 58, 175–189.

Fa, M., Diana, A., Carta, G., Cordeddu, L., Melis, M.P., Murru, E., Sogos, V., Banni, S., 2005. Incorporation and metabolism of c9,t11 and t10,c12 conjugated linoleic acid (CLA) isomers in rat brain. Biochim. Biophys. Acta - Mol. Cell Biol. Lipids 1736, 61–66.

Fang, S., Soczynska, J.K., Woldeyohannes, H.O., McIntyre, R.S., 2012. The association between comorbid anxiety disorders and pro-inflammatory cytokine levels in individuals with bipolar disorder. Univ. Toronto J. Undergrad. Life Sci. 6, 13–17.

Forsythe, P., Bienenstock, J., Kunze, W.A., 2014. Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease 817, 115–133.

Foster, J.A., McVey Neufeld, K.A., 2013. Gut-brain axis: How the microbiome influences anxiety and depression. Trends Neurosci. 36, 305–312.

Frank-Cannon, T.C., Alto, L.T., McAlpine, F.E., Tansey, M.G., 2009. Does neuroinflammation fan the flame in neurodegenerative diseases? Mol. Neurodegener. 4, 1–13.

Furusawa, Y., Obata, Y., Fukuda, S., Endo, T.A., Nakato, G., Takahashi, D., Nakanishi, Y., Uetake, C., Kato, K., Kato, T., Takahashi, M., Fukuda, N.N., Murakami, S., Miyauchi, E., Hino, S., Atarashi, K., Onawa, S., Fujimura, Y., Lockett, T., Clarke, J.M., Topping, D.L., Tomita, M., Hori, S., Ohara, O., Morita, T., Koseki, H., Kikuchi, J., Honda, K., Hase, K., Ohno, H., 2013. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature 504, 446–450.

Herter, C.A., Kendall, A.I., 1910. The influence of dietary alternations on the types of intestinal flora. J. Biol. Chem. 7, 203–236.

Honneffer, J.B., Minamoto, Y., Suchodolski, J.S., 2014. Microbiota alterations in acute and chronic gastrointestinal inflammation of cats and dogs. World J. Gastroenterol. 20, 16489–16497.

Jenkins, T.A., Nguyen, J.C.D., Polglaze, K.E., Bertrand, P.P., 2016. Influence of tryptophan and serotonin on mood and cognition with a possible role of the gut-brain axis. Nutrients 8, 1–15.

Jha, M.K., Park, D.H., Kook, H., Al., E., 2016. Metabolic Control of Glia-Mediated Neuroinflammation. Curr. ALZHEIMER Res. 13, 387–402.

Junej, L., Chu, D., Okubo, L., Et al, 1999. L-Theanine, a unique amino acid of green tea and its relaxation effect in humans. Trends Food Sci Technol 10, 199–204.

Kiecolt-Glaser, J.K., Belury, M.A., Andridge, R., Malarkey, W.B., Glaser, R., 2011. Omega-3 supplementation lowers inflammation and anxiety in medical students: A randomized controlled trial. Brain. Behav. Immun. 25, 1725–1734.

Lee, R., 2018. Anxiety. In: Rakel, D. (Ed.), Integrative Medicine. elesvier, pp. 46–52.

Levkovich, T., Poutahidis, T., Smillie, C., Varian, B.J., Ibrahim, Y.M., Lakritz, J.R., Alm, E.J., Erdman, S.E., 2013. Probiotic Bacteria Induce a “Glow of Health.” PLoS One 8, 1–11.

Lu, K., Gray, M.A., Oliver, C., Liley, D.T., Harrison, B.J., Bartholomeusz, C.F., Phan, K.L., Nathan, P.J., 2004. The acute effects of L-theanine in comparison with alprazolam on anticipatory anxiety in humans. Hum. Psychopharmacol. 19, 457–465.

Mayer, E.A., Knight, R., Mazmanian, S.K., Cryan, J.F., Tillisch, K., 2014. Gut Microbes and the Brain: Paradigm Shift in Neuroscience. J. Neurosci. 34, 15490–15496.

McKernan, D.P., Dennison, U., Gaszner, G., Cryan, J.F., Dinan, T.G., 2011. Enhanced peripheral toll-like receptor responses in psychosis: Further evidence of a pro-inflammatory phenotype. Transl. Psychiatry 1, e36-8.

Miller, A.H., Raison, C.L., 2017. Role of inflammation in depression. Nat Rev Immunol 16, 22–34.

Mulak, A., Bonaz, B., 2015. Brain-gut-microbiota axis in Parkinson's disease. World J. Gastroenterol. 21, 10609–10620.

Neufeld, K.-A.M., Kang, N., Bienenstock, J., Foster, J.A., 2011. Effects of intestinal microbiota on anxiety-like behavior. Commun. Integr. Biol. 4, 492–494.

Norman, H.J., 1909. Lactic acid bacilli in the treatment of melancholia. Br Med J 1, 1234–1235.

Notari, L., 2009. Stress in veterinary behavioural medicine. In: Horwitz, D., Mills, D. (Eds.), BSAVA Manual of Canine and Feline Behavioural Medicine. British Small Animal Veterinary Association, Gloucester, pp. 136–145.

O'Brien, S.M., Scott, L. V., Dinan, T.G., 2004. Cytokines: Abnormalities in major depression and implications for pharmacological treatment. Hum. Psychopharmacol. 19, 397–403.

Omran, Y. Al, Aziz, Q., 2014. Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease 817, 135–153.

Overall, K., 2013. Manual of Canine and Feline Behavioral Medicine. Elsevier Inc, St. Louis, MO.

Pace, T.W.W., Heim, C.M., 2011. A short review on the psychoneuroimmunology of posttraumatic stress disorder: From risk factors to medical comorbidities. Brain. Behav. Immun. 25, 6–13.

Peter, B.Y., Smith, A., 2015. Brain, Meet Gut 6–8.

Rapaport, M.H., Nierenberg, A.A., Schettler, P.J., Kinkead, B., Cardoos, A., Walker, R., Mischoulon, D., 2016. Inflammation as a predictive biomarker for response to omega-3 fatty acids in major depressive disorder: A proof-of-concept study. Mol. Psychiatry 21, 71–79.

Rooney, N.J., Gaines, S.A., Bradshaw, J.W.S., 2007. Behavioural and glucocorticoid responses of dogs (Canis familiaris) to kennelling: Investigating mitigation of stress by prior habituation. Physiol. Behav. 92, 847–854.

Schemann, M., Neunlist, M., 2004. The human enteric nervous system. Neurogastroenterol. Motil. 16, 55–59.

Schmidt, C., 2015. Thinking from the Gut The microbiome may. Nat. Publ. Gr. 312, s12–s15.

559 Sierra, A., Gottfried-Blackmore, A., Milner, T.A., McEwen, B.S., Bulloch, K., 2008. Steroid
560 hormone receptor expression and function in microglia. Glia 56, 659–674.
561 Smolek, T., Madari, A., Farbakova, J., Kandrac, O., Jadhav, S., Cente, M., Brezovakova, V.,
562 Novak, M., Zilka, N., 2016. Tau hyperphosphorylation in synaptosomes and
563 neuroinflammation are associated with canine cognitive impairment. J. Comp. Neurol. 524,
564 874–895.
565 Suchodolski, J.S., Camacho, J., Steiner, J.M., 2008. Analysis of bacterial diversity in the canine
566 duodenum, jejunum, ileum, and colon by comparative 16S rRNA gene analysis. FEMS
567 Microbiol. Ecol. 66, 567–578.
568 Szurszewski, J.H., 2003. Physiology of Mammalian Prevertebral Ganglia. Annu. Rev. Physiol. 43,
569 53–68.
570 Takeuchi, O., Akira, S., 2010. Pattern Recognition Receptors and Inflammation. Cell 140, 805–820.
571 Turner, E.H., Loftis, J.M., Blackwell, A.D., 2006. Serotonin a la carte: Supplementation with the
572 serotonin precursor 5-hydroxytryptophan. Pharmacol. Ther. 109, 325–338.
573 Venable, E., Bland, S., Holscher, H., Swanson, K., 2016. Effects of Air Travel Stress on the Canine
574 Microbiome: A Pilot Study. Int. J. Vet. Heal. Sci. Res. 132–139.
575 Wager-Smith, K., Markou, A., 2011. Depression: A repair response to stress-induced neuronal
576 microdamage that can grade into a chronic neuroinflammatory condition? Neurosci. Biobehav.
577 Rev. 35, 742–764.
578 Walker, J.K., Waran, N.K., Phillips, C.J.C., 2014. The effect of conspecific removal on the
579 behaviour and physiology of pair-housed shelter dogs. Appl. Anim. Behav. Sci. 158, 46–56.
580 Weiss, J.., 1972. Psychological factors in stress and disease. Sci. Am 226, 104–113.
581 Wohleb, E.S., McKim, D.B., Sheridan, J.F., Godbout, J.P., 2015. Monocyte trafficking to the brain
582 with stress and inflammation: A novel axis of immune-to-brain communication that influences
583 mood and behavior. Front. Neurosci. 9, 1–17.
584 Zhu, H., Qu, F., Zhu, L., 1993. Isolation of genomicDNAs from plants, fungi and bacteria using
585 benzylchloride. Nucleic Acids Res. 21, 5279–5280.
586 Zhu, X., Han, Y., Du, J., Liu, R., Jin, K., Yi, W., 2017. Microbiota-gut-brain axis and the central
587 nervous system. Oncotarget 8.
588
589

Figures captions

Fig. 1 Average values of the bacterial concentration expressed as log CFU/g related to *Bacteroides-Prevotella- Porphyromonas*. # Statistically different ($P \leq 0.05$) vs T0 (One-way ANOVA test). **Figures Legend: Control** = healthy dogs**, Therapy** = anxious dog treated with Relaxigen**, Placebo** = anxious dog treated without treatment; **T0** = starting point**; T1** = 30 days follow-up**; T2** = 60 days follow-up

Fig. 2 Average values of the bacterial concentration expressed as log CFU/g related to *Bifidobacterium* spp. * Statistically different ($P \leq 0.05$) vs Control group (One-way ANOVA test). **Figures Legend: Control** = healthy dogs**, Therapy** = anxious dog treated with Relaxigen**, Placebo** = anxious dog treated without treatment; **T0** = starting point**; T1** = 30 days follow-up**; T2** = 60 days follow-up

Fig. 3 Average values of the bacterial concentration expressed as log CFU/g related to *Clostridium coccoides- Eubacterium rectale* group. * Statistically different ($P \leq 0.05$) vs Control group; ╪ vs Placebo group; # vs T0 (One-way ANOVA test). **Figures Legend: Control** = healthy dogs**, Therapy** = anxious dog treated with Relaxigen**, Placebo** = anxious dog treated without treatment; **T0** = starting point**; T1** = 30 days follow-up**; T2** = 60 days follow-up

Fig. 4 Average values of the bacterial concentration expressed as log CFU/g related to *Enterobacteriaceae*. * Statistically different ($P \leq 0.05$) vs Control group; ╪ vs Placebo group (One-way ANOVA test). **Figures Legend: Control** = healthy dogs**, Therapy** = anxious dog treated with Relaxigen**, Placebo** = anxious dog treated without treatment; **T0** = starting point**; T1** = 30 days follow-up**; T2** = 60 days follow-up

Fig. 5 Average values of the bacterial concentration expressed as log CFU/g related to *Lactobacillus* spp. * Statistically different ($P \leq 0.05$) vs Control group; ╪ vs Placebo group; # vs T0 (One-way ANOVA test). **Figures Legend: Control** = healthy dogs**, Therapy** = anxious dog treated with Relaxigen**, Placebo** = anxious dog treated without treatment; **T0** = starting point**; T1** = 30 days follow-up**; T2** = 60 days follow-up

Fig. 6 Average values of the bacterial concentration expressed as log CFU/g related to *Staphylococcus* spp. **Figures Legend: Control** = healthy dogs**, Therapy** = anxious dog treated with Relaxigen**, Placebo** = anxious dog treated without treatment; **T0** = starting point**; T1** = 30 days follow-up**; T2** = 60 days follow-up