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Supplementation with nutraceutical Calmina® (BuonaPET) improves anxiety status in shelter dogs: a pilot study

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Abstract: Shelter dogs may experience long-lasting stressful living conditions. As a result, veterinarians should use every tactic at their disposal to enhance these animals' comfort and well-being. In the present study, we enrolled dogs with an anxious profile, administered with a food supplement, based on fish protein hydrolysate (PEPTIDYSS®), L-theanine, Griffonia simplicifolia, Lactobacillus Helveticus, Vitamin B6, Zinc and Magnesium. At the end of the 60-day treatment period, we documented for the first time a significant improvement in anxiety-based behaviors, evaluated by means an increase of problem-solving skills and interspecific sociability. Overall, our preliminary data shed light on the tantalizing role of Calmina® in animal shelter, with the purpose of improving the adoption chance for dogs, who experience emotional discomforts.

Key Words: dog behavior; stress; serotonin; cortisol; human-dog relationship; emotions

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Introduction

The quality of life at shelters is one of the primary issues surrounding the human-dog connection today, since dogs often live in conditions that do not respect their ethological needs, such as social interaction, exploration, physical activity and mental stimulation (d'Angelo et al., 2022). This can lead to stressful environments and experiencing negative emotions such as anxiety, fear, pain, and isolation; the effect of such an environment on dogs can also differ depending on the individual's past experiences and personality as well as kennel management (Corsetti et al., 2018; Iacopini & Gazzano, 2023). Stress represents a physiological response downstream the activation of the hypothalamic-pituitary-adrenal (HPA) axis which, in turn, regulates levels of glucocorticoid hormones, thus trying to adapt and keep on balance the neuroendocrine and behavioral system. In this respect, during stressful situations, the HPA axis causes the activation of the 'fight-or-flight' response (e.g. increased alertness and expression of stress behaviour) and the release of glucocorticoid hormones, including cortisol (Pariante et al., 2008). If stress fails to be addressed, it can result in serious health issues like anxiety, depression, burnout, and sleep disturbances (Kellendonk et al., 2002). Such detrimental effects on the emotional state becomes even worse in a time-dependent manner (Wells et al., 2002; Hiby et al., 2006). Together with glucocorticoid hormones, also serotonin (5-HT) is involved in the regulation of emotional state: is a neurotransmitter produced from tryptophan and is believed to be important in the aetiology and treatment of most behavioral disorders in dogs. Indeed, previous studies have reported that serotonin influences mood, cognition, and behavior, such as aggression, impulsivity, food selection, stimulation, sexual behavior, pain response, and emotional expressions (Ruddick et al., 2006; Cakiroglu et

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al., 2007; Berger et al., 2009; Bochis et al., 2022). Therefore, early intervention is needed to cope with the emotional alterations underlying the behavioral problems of dogs housed in kennels, thereby improving the chances of adoption (Gazzano et al., 2023). Growing evidence highlighted the importance of nutraceuticals based on alpha-casozepine, l-theanine, tryptophan, cannabidiol, cannabis, essential oil, melatonin and probiotics, in the treatment of behavioral disorders in dogs, since they have anxiolytic and antistress potentials, thus being regarded as an alternative to the conventional therapy (Sechi et al., 2017; Orlando, 2018; Tynes & Landsberg, 2021; Cannas et al., 2021; Ağan et al., 2022; Yu-Min et al., 2022; Sacchettino et al., 2023a). In addition, according to previous clinical cases, the administration of Calmina® in family dogs seems to reduce anxiety, stress and facilitate rehabilitation therapy in treating behavioral disorders (Sacchettino et al., 2023b). With this in mind, we sought to investigate in the present pilot study the potential role of daily administration of a Calmina® (BuonaPET) supplement in the anxiety management of nine mix-breed dogs, sheltered in an Italian kennel near Naples. This compound is enriched with different active ingredients: a protein hydrolysate from the by-products of sardine processing (PEPTIDYSS®), Zinc, Vitamin B6, L-theanine, Griffonia simplicifolia, Lactobacillus helveticus HA-122, and Magnesium, supplements that help promote the animal's state of calm and relaxation (Rahimi Niyyat et al., 2018; Araujoa et al., 2010; Titeaux et al., 2021; Yu-min et al., 2022).

Materials and Methods

Participants

All experimental protocols were approved by the Scientific Ethic Committee for Animal Experimentation on May 4th, 2023 (Reference number: PG/2023/0051881). The initial sampling involved 12 dogs. They were mixed-breed, both males (n = 6) and females (n = 6), all neutered, medium to large in size, according to the classification reported by Wilding (2018). Inclusion criteria: age two to eight years old, same commercial diet, time spent at least 12 months in shelter, and anxiety (value between 17-35) resulting from Evaluation of a Dog's Emotional Disorder (EDED Scale). As an exclusion criteria: the presence of organic and infectious diseases, and previous or ongoing behavioral rehabilitation (see Table 1).

Dogs	Age(years)	Gender	EDED value
Chicco	6	M	17
Serena	8	F	19
Blanco	6	M	17
Teo	6	M	19
Lady	8	F	24
Bella	6	F	17
Dino	6	M	18
Kocca	6	F	21
Sasà	2	M	17
Pupa	8	F	21
Spino	2	M	18
Sofy	2	F	21

Table 1. Characteristics of shelter dogs involved in the pilot study. The dogs involved were between two and eight years of age, fed the same commercial diet, had an anxious behavioural pattern resulting from the EDED (Evaluation of a Dog's Emotional Disorder) scale, and had to stay in the shelter for at least 12 months. All dogs were neutered. They had not undergone any previous or ongoing behavioural rehabilitation.

They were selected from the 80 guests of the Municipal Shelter of Pomigliano d'Arco (Naples), where they were housed after being caught on the territory by the local health authority, in order to limit the stray phenomenon. The structure of the kennel is organized in multiple boxes , based on intraspecific compatibility, to meet the social needs of the canine species. There is an open space as a walking area, in front of the boxes, where dogs could have a walk and interact with each other and with people.

The anxiety status was diagnosed by a veterinary behaviorist through a behavioral examination and using the EDED Scale of Pageat [as reported in d'Angelo et al., 2022], which allows the classification of dogs' behavior according to the presence/absence of centripetal and centrifugal behavior, and the expression of homeostasis or emotional disturbances. Social interactions, the ability to explore, and aggressiveness are centrifugal activities, whereas feeding, drinking, self-directed behavior, and sleep are centripetal activities. For each behavior considered, a specific score was attributed to each subject. Each dog then obtained a total score, which indicated its general emotional state. The dogs enrolled had a score from 17 to 24, corresponding to an anxious state, in particular in the presence of new stimuli, new people and/or loud noises. The animals underwent a complete blood test, which also included the evaluation of thyroid function, and protein electrophoresis, to rule out any co-morbidities, potentially related to behavioral issues (Camps et al., 2019; Amat et al., 2023). Three of them (Lady, Spino, Dino) showed organic pathologies; therefore, from an initial population of 12 animals, we administered the supplement to 9 healthy dog.

Procedures

The research was structured over the 2-month period, as follows:

- dogs evaluations: behavioral and biochemical examinations, cortisol and serotonin analysis;
- daily administration of the nutraceutical Calmina® in dogs enrolled, according to the dosage/weight listed on the package, for 60 days (T_0);
- on the 60th day, behavioral follow-up by veterinarian expert in animal behavior and new blood sampling for cortisol and serotonin analysis (T_{60}).

Behavioral assesment

To assess behavioral changes in dogs assuming Calmina®, the veterinary behaviorist tested the nine enrolled dogs at T_0 and T_{60} using a modified temperament test (Valsecchi et al., 2011), by observation directly lasting 5 minutes per dog, between 8.30 and 10.30 a.m. before eating. The veterinary behaviorist (tester) who performed the behavioral assessment did not participate in any sampling collections. Behavioral assesment covered 5 behavioral domains: Approaching the kennel, Entering the kennel, Interspecific interaction (physical contact), the "come" command, Problem solving. Higher scores corresponded to a friendlier and more relaxed behaviour of the animal. For a description of the 5 domains, see Supplementary Materials.

Sample Collection and Cortisol- Serotonin Measurement in Dogs

Blood sampling was performed between 8:30 am and 10:30 am before eating, as part of the health monitoring procedures routinely carried out by the kennel. A small patch of hair was shaved from the thoracic limb and topical anaesthesia [Eutectic Mixture of Local Anaesthetics (EMLA™) cream] applied to the area before collection of a 5 ml blood sample from the saphenous vein.

After the sampling, the blood samples were placed in a polystyrene container with ice and transported to the laboratory, where they were promptly centrifuged at 3000 rpm in a centrifuge for 10 min to obtain the serum for biochemistry analysis, cortisol and serotonin determination.

The samples obtained were stored at -20° C and then proceeded. Cortisol and serotonin levels were determined by immunoassay using the commercially available cortisol and serotonin ST/5-HT (5-hydroxytryptamine) canine kit (FineTest, Wuhan China), according to the manufacturer's indications.

Statistical Analysis

Data were analysed through paired Student t-test in GraphPad Prism 10 software (La Jolla, California). Results were considered statistically significant for $p < 0.05$.

Results

Here, we evaluated the serum levels of cortisol and serotonin in the dogs supplemented for two months with Calmina®: our data showed no main effect of the nutraceutical (T_{60}), when compared to the untreated (T_0) condition ($p > 0.05$, paired Student's t test), as reported in figure 1.

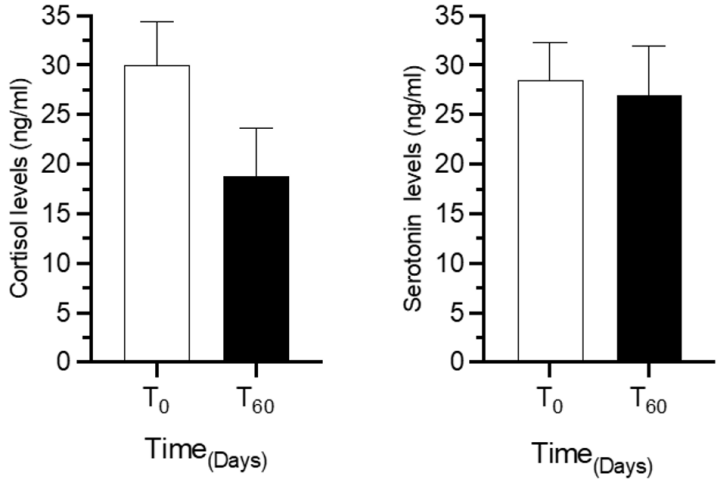

Figure 1. Effect Calmina® supplementation on the serum cortisol and serotonin levels in dogs. Cortisol and serotonin levels (ng/ml) following two-month Calmina® treatment. p > 0.05, compared to T_0 group (paired Student's t test). All values are expressed as mean ± SEM.

Then, to assess behavioral changes in dogs assuming Calmina®, we analyzed the behavioral observation score resulting in the modified temperament test at T_0 and T_{60}, on domains:

1) Approaching the kennel
2) Entering the kennel
3) Interspecific interaction (physical contact)
4) The "come" command
5) Problem solving.

The statistics were made by paired Student t-test. Results were considered statistically significant for $p < 0.05$. The data of these behavioral observations allow us to understand how sociability and the ability to solve a problem increased (Figure 2).

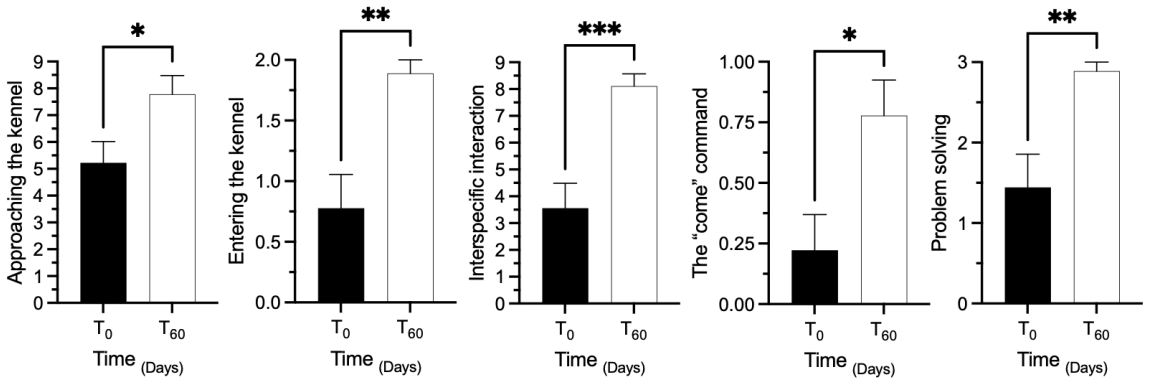

Figure 2. Behavioral observation score. Behavioral changes in dogs assuming Calmina® at modified temperament test performed by the behaviorist at T_0 and T_{60}. *, **, ***: $p < 0.05$, 0.01, 0.001, respectively; paired Student t-test.

Discussion

The results of our pilot study allowed us to observe an improvement in anxious behaviors in dogs treated with Calmina® for the 60-day supplementation, although serum cortisol and serotonin levels did not change significantly.

In Italy, there are several laws on stray dogs, including the regional regulation (Regional Law 11 April 2019, n. 3.) that obliges the shelter to employ the figure of the dog trainer, since consider that a well-planned and tailored training programme can increase emotional and cognitive abilities in dogs, and this represent a crucial factor in restoring a better human-dog relationship (d'Angelo et al., 2022). However, when dogs have altered levels of anxiety, their abilities to learn news skills and behavior, or experience positive emotions are impaired, making the behavioral rehabilitation program more complicated (Sacchettino et al., 2023). Shelter dogs frequently experience negative emotions due to living in socially unsatisfactory and spatially restricted environments; such conditions generally result in increased arousal, intraspecific issues and instability in dogs, thus making them less suitable for adoption (Breeda et al., 1999; d'Ingeo et al., 2021). In our results, the dogs improved on sociability, evaluated by the domains "Approaching the kennel", "Entering the kennel", "Interspecific interaction (physical contact)" and the "Come" command, showing themselves more prone to social interactions. Dogs' adoptability in shelters could be enhanced by this increased social friendliness, on account of the adopters believe that animals with adequate social behavior are more approachable and less aggressive (Wright et al., 2007). Despite the lack of information on the influence of social interaction on adoption success, dogs that typically act withdrawn, antisocial, or fearful have a lower chance of being adopted (Tuber et al., 1999; Protopopova et al., 2014). Moreover, the reduction of anxiety should also be considered from a canine welfare and life impact perspective; in fact, Dreschel (2010) showed that dogs with extreme fear directed by the stranger hesitated in a shorter lifespan of six months than dogs without fear directed by the stranger. In addition, fearful dogs may show more discomfort in a hospital situation where they are exposed to and handled by strangers, negatively impacting their physical and emotional health (Dreschel et al., 2010). Therefore, all canine therapeutic interventions – including those based on nutraceuticals – could be useful to improve the level of interspecific social interaction in dogs housed in kennels. Our dogs have significantly improved in the 'Problem-solving' domain, demonstrating their ability to stay on a task by using their olfactory intelligence. In our view, this improvement is related to a reduction of the anxiety's state, as this emotional discomfort in dogs can manifest in various ways, such as decrease of appetite and of olfactory – exploratory activity, hypervigilance, avoidance, hiding, escape attempts (Gazzano et al., 2020; Landsberg, 2023). In line with this, previous research

indicated that intermediate degrees of arousal are essential for optimal performance; conversely, too low or high arousal levels (as in anxiety status) can adversely affect and hinder performance (Mills, 2010; Bray et al., 2015). Furthermore, in recent study sniffing is potentially defined as an explicit cognitive behavior (Kokocinska-Kusiak et al., 2021), which requires concentration and an intermediate level of arousal. It's no surprise, since the anxiety and the concentration are negatively related: in human medicine, anxiety symptoms in adolescents are linked to poor concentration, influencing on their school performance over time (Leigh et al., 2021). Even in dogs, as shown by d'Angelo and collaborators in (2022) the ability to remain concentrate on olfactory research is related to the state of emotional activation (arousal); therefore, the improvement of olfactory behavior could depend on reducing anxiety levels and on achieving an appropriate emotional level for the learning process.

Regarding serum cortisol levels, our research shows a trend – not statistically significant – of decreasing levels in all enrolled dogs. It is possible that nutraceuticals may have contributed to reduced cortisol levels and improved stress resilience in dogs, as reported by Sechi and colleguas in 2017. In addition, a further explanation is that even though blood cortisol measurement is a very precise method of stress assessment, the invasive nature of sampling could lead to temporary spikes in cortisol (Hanna et al., 2019; Marza et al., 2024), which Calmina® supplementation may not have significantly prevented. Furthermore, environmental stress may have played a role in sampling: the blood samples were taken at the kennel's veterinary clinic, place that was not conducive to the emotional comfort of dogs (Gazzano et al., 2025). Lastly, the small number of dogs involved may not have been sufficient for a statistically significant change in blood cortisol levels.

Regarding serotonin levels, despite the observed improvements in behaviors, no significant change in canine serum was observed. Previous research by De Napoli and Dodman (2000) reported an improvement in the aggressiveness of dogs with tryptophan supplementation, a precursor of serotonin. Although Calmina® is enriched in griffonia as a source of tryptophan, our results do not confirm a significant variation in serotonin levels, in the canine state of anxiety. One possible explanation is that we investigated a different disorder (aggression vs anxiety), and this would explain the discordant results, since other neurotransmitter systems – besides the serotonergic system – may be involved in anxiety (i.e. GABAergic System). Nevertheless, also Riggio and colleagues observed no association between serum levels of tryptophan and serotonin in their study on shelter dogs (Riggio et al., 2021). Whereas the blood-brain barrier controls the flow of serotonin in an opposite way to that of tryptophan – from peripheral circulation to central circulation – it could help explain the negative results. Despite the continuing desire to try to draw a link between behaviour, peripheral serotonin level and tryptophan, future research is needed to explain their mechanism and correlation. According to these findings, behavioral assessment could be a more accurate way to measure canine stress than just relying on hormonal or physiological parameters. In addition, it is important to consider the personality of dogs with behavioral dysfunction so that an individualized rehabilitation plan can be implemented, taking into account context and relationships, together with nutrition, gut-brain axis and reproductive status (Tynes & Landsberg, 2021; Landsberg, 2023; Ogi et al., 2024; Sacchettino et al., 2025).

Conclusions

The results of this pilot study showed that supplementation with the nutraceutical Calmina® (BuonaPET) in shelter dogs improved anxiety-related behaviors and sociability, although serum cortisol and serotonin levels did not show statistically significant changes. It's worth noting that there were no behavioral programs employed during the supplementation period, which highlights the effects of the nutraceutical without any additional variables. Supplementing nutraceuticals can be beneficial in protecting the emotional well-being of dogs in kennels and increasing their sociability. Further research is recommended to investigate the role of nutraceuticals on anxiety behaviors in sheltered dogs and the neurotransmitter pathways targeted by nutraceuticals.

Limitations

Despite the encouraging behavioral results of this pilot study, it is plausible that a few limitations might have influenced the results obtained. To begin with, we recognize that our study involved a small number of patients, due to the tightening factors we have chosen as inclusion criteria in the recruitment and in the analyses performed in the kennel's environment; indeed, we decided to exclude dogs that had signs of organic or infectious disease, which could have altered the results. In addition, this pilot study covered two months of supplementation: this short interval may not have been sufficient to induce neurotransmitter changes. In conclusion, the lack of a control group without treatment, as we evaluated variations within the same sample group. Further studies are needed to investigate the relationship between dysfunctional behaviour and nutraceuticals in kennelled dogs. The effect of nutraceuticals on behavioural modification should also be investigated in conjunction with the rehabilitation programme.

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Conflicts of Interest: The authors state that they received the commercial product from the company Buona Spa Società Benefit, Via Alfieri, 8, 50019 Sesto Fiorentino (FI).

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L'integrazione con il nutraceutico Calmina® (BuonaPET) migliora lo stato d'ansia nei cani dei rifugi: uno studio pilota

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Sintesi

I cani dei rifugi possono vivere in condizioni di stress prolungate. Di conseguenza, i veterinari dovrebbero utilizzare ogni strategia a loro disposizione per migliorare il comfort e il benessere di questi animali. Nel presente studio, abbiamo arruolato cani con un profilo ansioso, ai quali è stato somministrato un integratore alimentare a base di idrolizzato proteico di pesce (PEPTIDYSS®), L-teanina, Griffonia simplicifolia, Lactobacillus Helveticus, Vitamina B6, Zinco e Magnesio. Al termine del periodo di trattamento di 60 giorni, abbiamo documentato per la prima volta un significativo miglioramento dei comportamenti ansiosi, valutato attraverso un aumento delle capacità di problem-solving e della socialità interspecifica. Nel complesso, i nostri dati preliminari hanno fatto luce sul ruolo allettante di Calmina® nei rifugi per animali, con l'obiettivo di migliorare le possibilità di adozione per i cani che sperimentano disagio emotivo.

Supplementary Material

Behavioral assesment covered 5 behavioral domains, described as follows:

1) Approaching the kennel: the observer quietly approaches the kennel with a neutral posture, avoiding direct eye contact, and stops a few centimeters from the fence. Postures and behaviors were labeled for simplicity as: friendly (tail wagging, nonaggressive barking, exuberant, and/or calm approach seeking contact with the observer), neutral (i.e., the dog holds still, showing neither threatening nor friendly behaviors), fearful (a crouched posture, including ears, and tails held low, avoiding eye gaze, shaking, whimpering), or threatening (barking, growling, lunging towards the mesh, stiff posture, piloerection, etc.). This behavior was observed for 3 minutes.

Score

Friendly and calm dog, approaching the tester asking for contact = 9;
Friendly dog, still, inside the kennel, not asking for contact = 8
Approaching exuberant and excited dog, hyperactive = 7
Neutral dog, calmly approaching the tester = 6
Neutral and still dog, only looking at the tester or barking = 5
Neutral dog avoiding contact, moving away from the tester = 4
Fearful dog, approaching the tester in low posture = 3
Fearful dog, still, in the center of the kennel= 2
Fearful dog avoiding the tester, moving away, and/or hiding in its shed/hut =1
Aggressive and threatening dog = 0

2)Entering the kennel: the observer walks into the kennel and closes the door. For 30 seconds the observer stands still, arms held loosely along the side, ignoring the dog completely, taking care not to cross its' eye gaze.

Score

The dog approaches the tester = 2
The dog stands still = 1
The dog goes away from the tester = 0

3) Interspecific interaction (physical contact): the observer stands still in the kennel, bends forward slightly, calling the dog in a gentle, relaxed manner. If there is no response, the observer may hold his/her hand out toward the dog, palm-side up, and even seek physical contact. The dog's behavior in the subsequent 30 seconds is observed.

Score

Friendly and calm dog, approaching the tester asking for contact = 9;
Friendly dog, still, inside the kennel, not asking for contact = 8
Approaching exuberant and excited dog, hyperactive = 7
Neutral dog, calmly approaching the tester = 6
Neutral and still dog, only looking at the tester or barking = 5
Neutral dog avoiding contact, moving away from the tester = 4
Fearful dog, approaching the tester in low posture = 3
Fearful dog, still, in the center of the kennel= 2
Fearful dog avoiding the tester, moving away, and/or hiding in its shed/hut =1
Aggressive and threatening dog = 0

4) "Come" command: the dog is off leash, the observer stands a minimum of 5 m from the dog, and calls the dog with a calm voice for approximately 30 seconds. If the dog is reticent, the observer may kneel down and call the dog once more holding his/her hand out, palm up.

Score:

The dog goes toward the tester after being called = 1
The dog does not follow the command = 0

5) Problem solving: the dog is off leash, the observer gives a titbit of food to the dog to evaluate its interest in the food reward; the observer approaches the dog, shows a second titbit, and making sure that the dog is still watching, places it on the floor. The observer moves away and notes whether the dog explores the object and attempts to retrieve the titbit within 30 seconds.

Score

The dog accomplishes the task = 3
The dog interacts with the box but does not accomplish the task = 2
The dog is interested in the food but looks at the tester not interacting = 1
The dog is not interested in the food or in the task = 0.