

Hematological Aspects on Dogs with Apparent Dysbiosis after *Bacillus Subtilis*, *Bacillus Licheniformis* and *Pediococcus Acidilactici* Probiotic Administration- Pilot Study

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Bulletin UASVM Veterinary Medicine 77(2)/2020
Print ISSN 1843-5270; Electronic ISSN 1843-5378
doi:10.15835/buasvmcn-vm:2020.0018

Abstract

The aim of the study was to assess the effect of a probiotic product based on *Bacillus subtilis*, *Bacillus licheniformis* and *Pediococcus acidilactici* on hematological parameters for dogs with apparent intestinal dysbiosis. Also, a comparison between the results obtained before and after the treatment was made, in order to establish if the probiotic treatment is able to improve the hematological results for dogs with apparent dysbiosis. A total number of 5 dogs, aged between 7 months and 6 years old were clinically evaluated for gastro-intestinal problems that lead to the suspicion of apparent intestinal dysbiosis. The probiotic administration respected the producer recommendations and it was given to the dogs together with their usual food, one capsule/day, 30 days consecutively. On the first and 31 days of the study, blood samples were collected and hematological parameters were evaluated. A heterogeneity in the hematological results was observed. However, when results from day 0 were compared with results obtained in day 31, the variance was considered to be physiological. Also, the small differences between the values before and after the treatment can not be attributed only to the probiotic treatment. The results obtained regarding the dynamics of the hematological parameters provided data that sustain the benefic effect of this probiotic combination, without a direct impact on hematological parameters.

Keywords: probiotic, dysbiosis, canine, *Bacillus subtilis*, *Bacillus licheniformis*, *Pediococcus acidilactici*

Introduction

Gastro-intestinal (GI) microbiome can be defined as a complex group formed from bacteria, arcanobacteria, fungi, protozoa and viruses that are able to colonize the mammals GI tract. GI microbiome is directly involved in some vital physiological processes (maintaining homeostasis, metabolic processes or immunological defense) (Barko *et al.*, 2018).

If the GI microbiome structure is modified, the host may show signs of illness (Barko *et al.*,

2018). One of those pathologies is represented by dysbiosis. Dysbiosis is defined as a state represented by some modification on the normal composition of the microbiome that is associated with other pathologies that affect the host's microbial homeostasis. (Honneffer *et al.*, 2014). Moreover, dysbiosis is characterized by a decrease of the number and bacteria diversity (Barko *et al.*, 2018). Also, there are proofs that it is a link between dysbiosis and GI tract pathologies like inflammatory bowel syndrome, granulomatous

colitis and irritable bowel syndrome (Honneffer *et al.*, 2014).

All those pathologies can affect the host's normal lifestyle. Because it is a complex problem, there is not available yet a treatment that address all the symptoms. However, probiotics administered to dogs with dysbiosis can produce an improvement on the health state. Probiotics are capable to produce benefic effects to the host without changing the permanent structure of the microbiome. This phenomenon is possible by temporary colonization of the intestine (Ceapa *et al.*, 2013; Barko *et al.*, 2018).

Probiotics are used since 20th century. Back then was reported a case of autoimmune arthritis that improved after probiotic treatment with some strains represented by *Streptococcus lacticus* and *Bacillus bulgaricus* (Warden, 1909; Barko *et al.*, 2018). In veterinary medicine, several strains of probiotics are used. However, some of them are considered to be more resistant and to produce a higher effect. Generally, sporulated bacteria are considered to have a higher resistance. Bacteria's spores are a conservation form, being produced by bacteria as a defense mechanism and, moreover, as a conservation form when the environmental conditions are not suitable for surviving. By this mechanism, bacteria are able to assure their survival a long time in some conditions that, in a normal way, would destroy the vegetative form of the bacterium (Nicholson *et al.*, 2000; Cutting, 2011).

The aim of the present study was to analyze the influence of a probiotic product with *Bacillus subtilis*, *Bacillus licheniformis* and *Pediococcus acidilactici* on hematological parameters on dogs with apparent dysbiosis. Also, a comparison between the results obtained before and after the treatment was made, in order to establish if the probiotic treatment is able to improve the hematological results for dogs with apparent dysbiosis.

Materials and Methods

The study was conducted at the University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca (on physiology and internal medicine departments) between May 2019 and June 2019. The study was approved by the Institutional Bioethics Committee by Decision no 130/20 December 2018.

Study design

A total of 5 adult dogs were enrolled in the study after a clinical examination, based on inclusion/exclusion criteria. Inclusion criteria were represented by GI manifestations representative for dysbiosis (diarrhea, vomiting and/or antibiotic treatment). Exclusion criteria were represented by the intestinal parasitism, chronic renal or hepatic failure. On day 0 of the study a full clinical examination was performed and blood was collected on EDTA in order to perform the hematological analysis. Between day 1 and 30 of the study the probiotic product containing *B. subtilis* HU58, *B.licheniformis* SL307, *P. acidilactici*, liver powder (FidoSpore® supplied by Microbiome Labs, LLC) was administered to the dogs, by the owner, respecting the manufacturer recommendations (daily, one capsule per day, during 30 days). On day 31 of the study the clinical examination as a follow-up was performed and the blood samples were collected again (one sample for each individual, on EDTA) in order to perform the hematological analysis.

Study population

All the 5 dogs were enrolled in the study after the clinical examination that revealed the meeting of the inclusion criteria. Also, all the dogs were enrolled in the study after their owners understood all the procedures and after that they signed a voluntary informed consent form. The study population was formed from P-01 (Labrador, 7 month, male with diarrhea and vomiting), P-02 (Akita Inu, 5 years and 9 months, female, with diarrhea and pancreatitis), P-03 (Golden retriever, 4 years, male, with dermatitis treated with antibiotics), P-04 (Boxer, 2 years, male, recurrent diarrhea induced by stress) and P-05 (Bucovina Shepperd, 4 years and 5 months, male, with recurrent diarrhea).

Treatment

The tested probiotic product was presented as capsules. The composition of the product was represented by the following bacterial strains: *B. subtilis* HU58, *B.licheniformis* SL307, *P. acidilactici*, plus an addition of liver powder (used in order to improve the taste of the product and to facilitate the ingestion). The capsules were administered during 30 days, one capsule per day, together with the usual food of the dogs.

Investigations

Clinical examination was performed using the great semiological methods. Therefore, the res-

piratory rate, cardiac frequency and temperature were registered, together with all the observations made on the systems and the values obtained were compared with the reference intervals from Merck Veterinary Manual (Merck *et al.*, 2016).

Hematological assessments were made using blood collected on anticoagulant (EDTA). ABA-CUS Junior VET was used in order to make the determinations, for the samples collected on day 0 and for the ones collected on day 31. Hematocrit (HCT), Hemoglobin (HBG), Total Red Blood Cells (RBC), Mean corpuscular volume (MCV), Mean corpuscular Hemoglobin (MCH), Mean Corpuscu-

lar Hemoglobin Concentration (MCHC) and Total White Blood Cells (WBC) were assessed using the semi-automatic machine (Abacus Junior Vet User's Manual). Also, a differential white cell count was performed using a blood smear and counting cells using the microscope.

Statistical analysis

The data were analyzed using GraphPad Prism 8.0 software. Descriptive statistics was performed for all the data obtained. Also, T test was performed in order to compare the data from day 0 and those from day 31. Significance was set at $P < 0.05$.

Table 1. Statically analysis before and after the treatment

Parameter	Descriptive statistics						T test	
	Min	Max	Mean	St Dev	St error of mean	CV (%)	p value	Sign
HCT pre-A	38.93	43.28	41.67	1.7070	0.764	4.10	0.2273	ns
HCT post-A	36.04	42.31	39.26	3.0460	1.362	7.76		
HGB pre-A	18.20	20.50	19.38	0.9524	0.425	4.91	0.2400	ns
HGB post-A	16.00	19.80	18.02	1.6900	0.755	9.38		
RBC pre-A	5.75	8.04	6.65	0.8987	0.402	13.50	0.6023	ns
RBC post-A	5.48	7.46	6.42	0.7946	0.355	12.30		
MCV pre-A	54.00	69.00	63.4	6.1070	2.731	9.63	0.0418	s*
MCV post-A	53.00	66.00	61	4.9500	2.214	8.11		
MCH pre-A	22.50	33.70	29.46	3.2840	1.469	11.15	0.0400	s*
MCH post-A	22.70	30.20	27.14	3.2840	1.468	12.09		
MCHC pre-A	44.70	48.90	46.6	1.7580	0.786	3.77	0.3760	ns
MCHC post-A	44.30	47.10	45.88	1.0920	0.488	2.38		
WBC pre-A	6.83	17.13	11.39	3.7500	1.670	32.97	0.8866	ns
WBC post-A	7.08	14.97	11.24	3.0750	1.375	27.35		
N pre-A	38.00	64.00	54.60	11.2600	5.036	20.62	0.0169	s*
N post-A	62.00	75.00	68	5.2440	2.345	7.71		
E pre-A	4.00	10.00	6.2	2.3870	1.068	38.51	0.7765	ns
E post-A	1.00	11.00	5.4	4.3930	1.965	81.36		
B pre-A	0.00	0.00	0	0.0000	0.000	0.00	-	-
B post-A	0.00	0.00	0	0.0000	0.000	0.00		
M pre-A	8.00	14.00	10.2	2.3870	1.068	23.41	0.8634 0.0315	ns s*
M post-A	4.00	14.00	10.6	3.9750	1.778	37.50		
L pre-A	21.00	47.00	29	11.2000	5.010	38.63		
L post-A	10.00	22.00	15.8	5.4950	2.458	34.79		

pre-A- determination made on day 0; post-A- determination made on day 31; N- neutrophils; E- eosinophils; B- basophils; M- monocytes; L- lymphocytes;

s*- statistically significant ($P < 0.05$); ns- without statistical significance ($P > 0.05$)

Results and Discussion

For all the dogs the clinical examination showed clinical signs of apparent dysbiosis. However, the 30 days follow-up showed a great improvement in the GI manifestation for all the dogs. The respiratory rate, hearth frequency and temperature were within normal limits on both examinations, for the whole population.

Hematological results showed a variance between the determinations (Table 1, Figure 1, Figure 2). From a statistical point of view, in case of neutrophils and lymphocytes the standard deviation and standard error of mean registered slightly high values (N pre-A St Dev: 11.2600 and St error of mean: 5.036; L pre-A St Dev: 11.2000 and St error of mean: 5.010). These results could be assigned to the study group heterogeneity. For some parameters, the results obtained on day 31 showed an improvement. Moreover, when compared, MCV, MCH, neutrophils and lymphocytes, have a similar evolution before and after the treatment (Fig 3, Fig 4, Fig 5, Fig 6).

For all the 5 dogs enrolled in the study the preliminary clinical exam showed signs like diarrhea, vomituration or skin problems, all being the reason to include the patients as subjects in the present study. Regarding the hematological parameters, heterogeneity was observed in all 5 cases. However, the differences recorded were more or less included in the physiological interval. Moreover, the heterogeneity of hematological results can be assigned to the heterogeneity of the study population.

Once the spores have been ingested, they need to resist the stomach barriers but in the other hand needs to be metabolically activated in the intestine. That's why it is of interest to understand the life cycle of *Bacillus* in the gut together with the action of *P. acidilactici*. In their review Bernardeau *et al.* (2017) are studying the main characteristics of beneficial *Bacillus* spores and vegetative cells (Bernardeau *et al.*, 2017).

Bacillus spores once ingested are submitted to the gastrointestinal transit (enzymes, bile salts, peristaltic movements). They have a high tolerance for gastric acidity due to their structure. In the intestine, spores will germinate in presence of nutrients, lysozyme, salts, high pressures and cationic surfactants (dodecylamine). These agents will bind to the spore's receptors and will release a concentration of dipicolinic acid (DPA). This

mechanism will trigger the entrance of water in the spore core. Subsequently rehydrated spores will start their germination. These processes lead to the presence of live *Bacillus* bacteria in the gut. Vegetative cells will proliferate and replicate. Finally, *Bacillus* will be present in stool and release in the environment (Bernardeau *et al.*, 2017). The rate, the location and velocity of germination are important factors to take in consideration as potential probiotics (Bernardeau *et al.*, 2017).

It is known that *B. subtilis* may not produce any changes in hematological parameters for animals. Hong *et al.* (2008) administered this bacterium strain to rabbits without any significant modification on the hematological results (Hong *et al.*, 2008). Regarding the results obtained for dogs, Szakacs *et al.* (2019) showed that the combination between *Bacillus* strains and *P. acidilactici* does not produce important modifications at the level of hematological parameters when it is administered to healthy dogs (Szakacs *et al.*, 2019).

A hematological assessment is a very common procedure used in clinics. Non-biased results are of a great importance in establishing a correct diagnostic. Because probiotics can have a systemic impact, by their complex mechanism of action, some parameters can be influenced by the treatment (Hong *et al.*, 2008). *Bacillus* spores carry capacity to survive to intestinal transit. (Cutting, 2011). Studies have proved that *Bacillus subtilis* spores can germinate in the small intestine, grow, proliferate and finally re-sporulate. *Bacillus* spores in feces can reach 10^4 spores/gram (Cutting, 2011). Studies in mice models have shown an increase of immune system due to administration of germinating spores (Cutting, 2011). Moreover, works shown that vegetative cells play a greater role in immune response than spores. *Bacillus* can have different immune responses according to the strains and the host and other factors such as the stage of *Bacillus* (Bernardeau *et al.*, 2017). By secreting antimicrobials like coagulant, amicoumacin and subtilisin *Bacillus* species is beneficial for the health. In fact, this property provides ability for the host to compete against pathogenic microorganisms (Cutting, 2011). Furthermore, *Bacillus* produces enzymes such as amylase, glucoamylase, protease, pectinase, and cellulase. *Bacillus* produces also vitamins such as riboflavin, cobalamin, inositol and carotenoids (Elshagabee *et al.*, 2017). As it can be seen, the

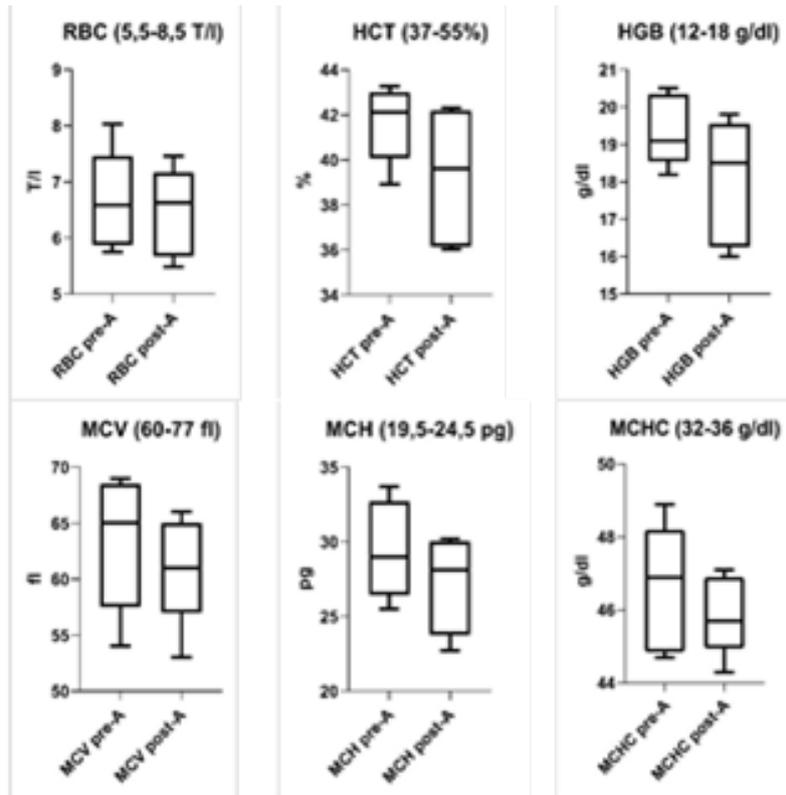


Figure 1. Red blood cells parameters- comparison before and after the treatment

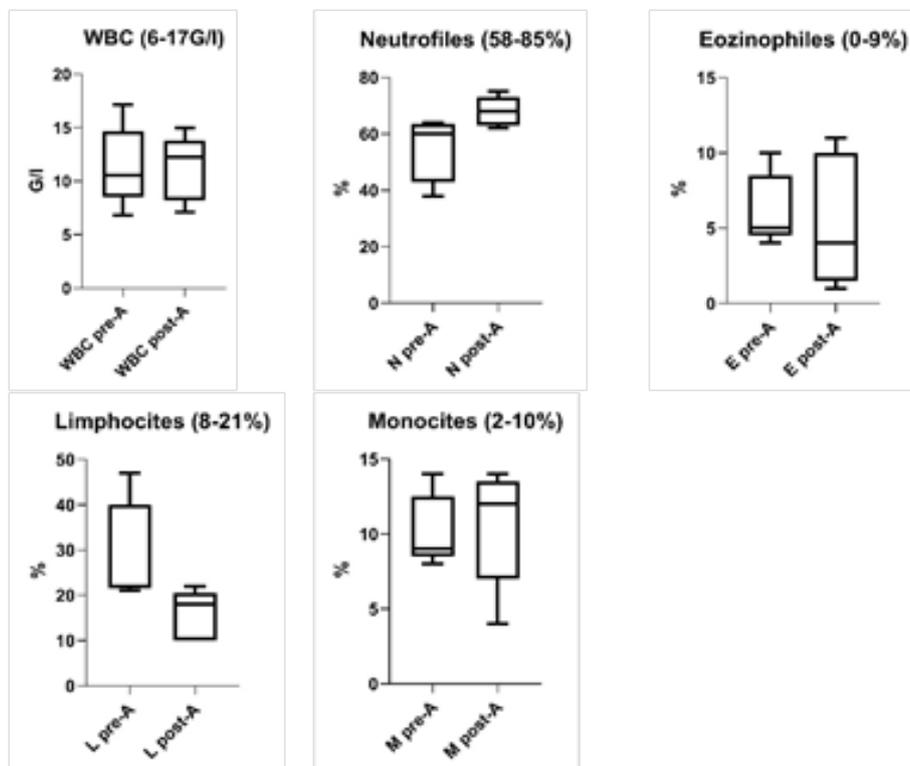


Figure 2. White blood cells parameters- comparison before and after the treatment

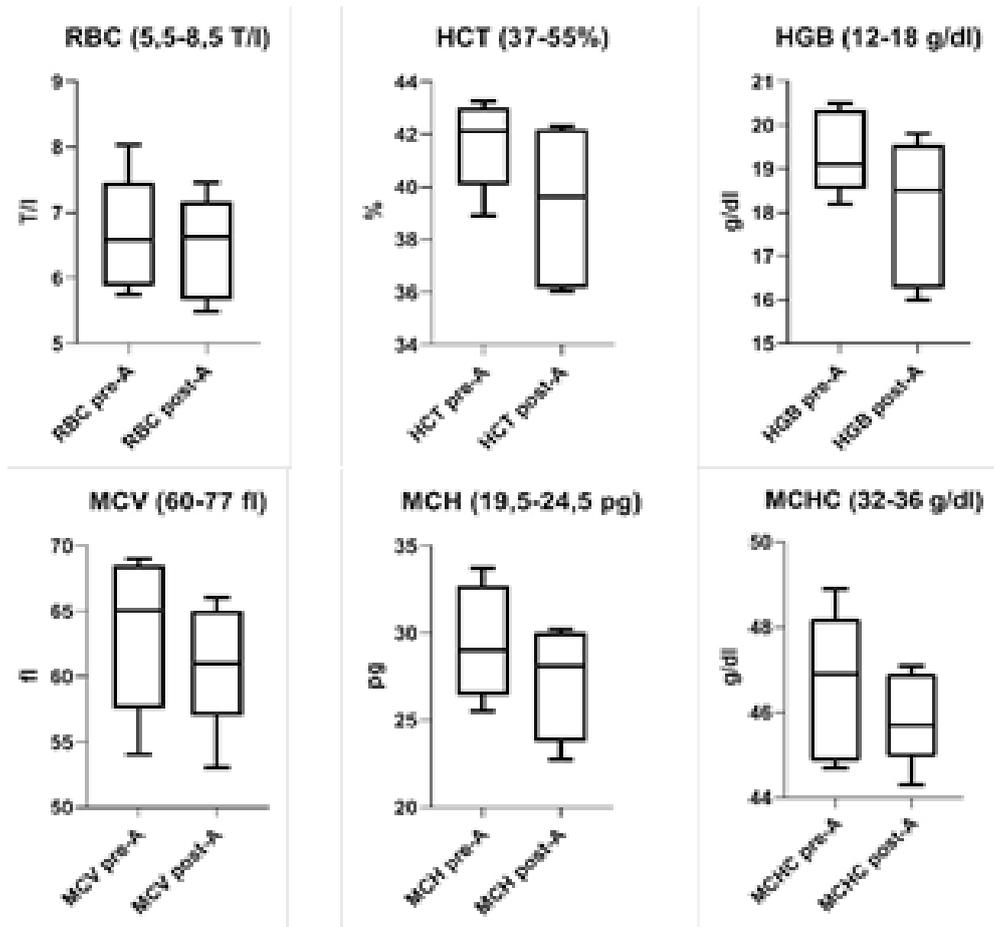


Figure 3. MCV Evolution before and after the treatment

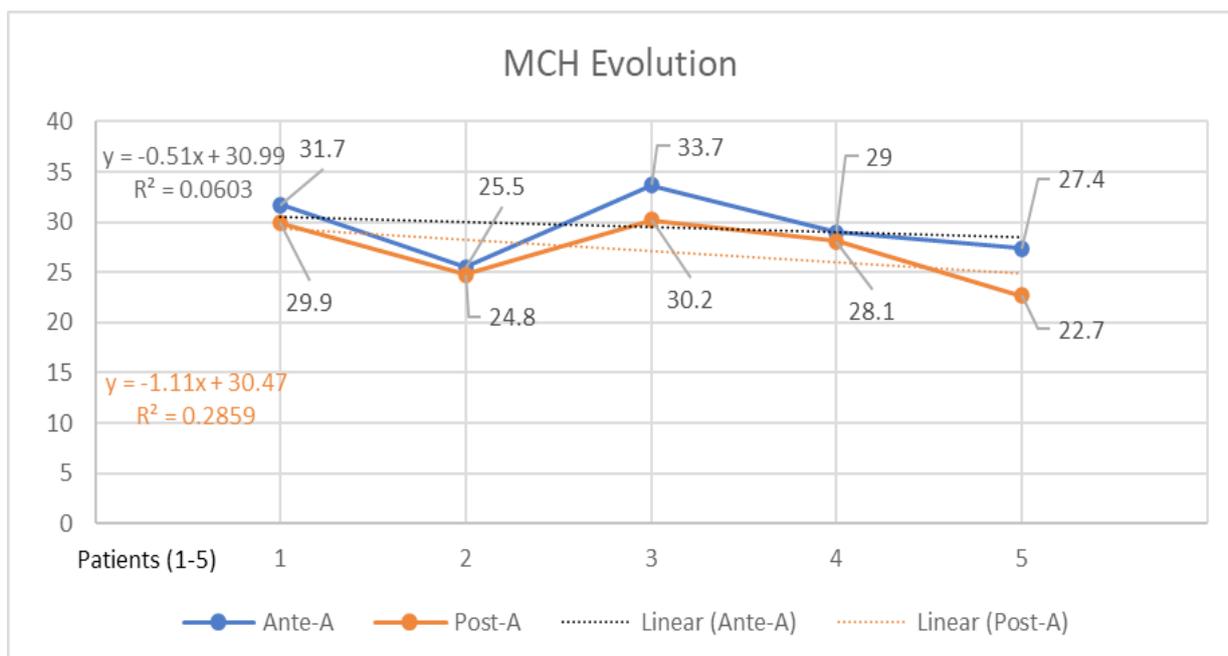


Figure 4. MCH Evolution before and after the treatment

complex mechanism of action is working at a systemic level. Usually, hematological parameters are assessed in order to clarify some dilemmas regarding pathologies also with systemic influence. If a treatment is able to influence the hematological

parameters, the interpretation of results can be biased and the diagnostic misunderstood. That can be considered a strong argument for establishing if a potential treatment is safe to use, without any influence on the assessed parameters.

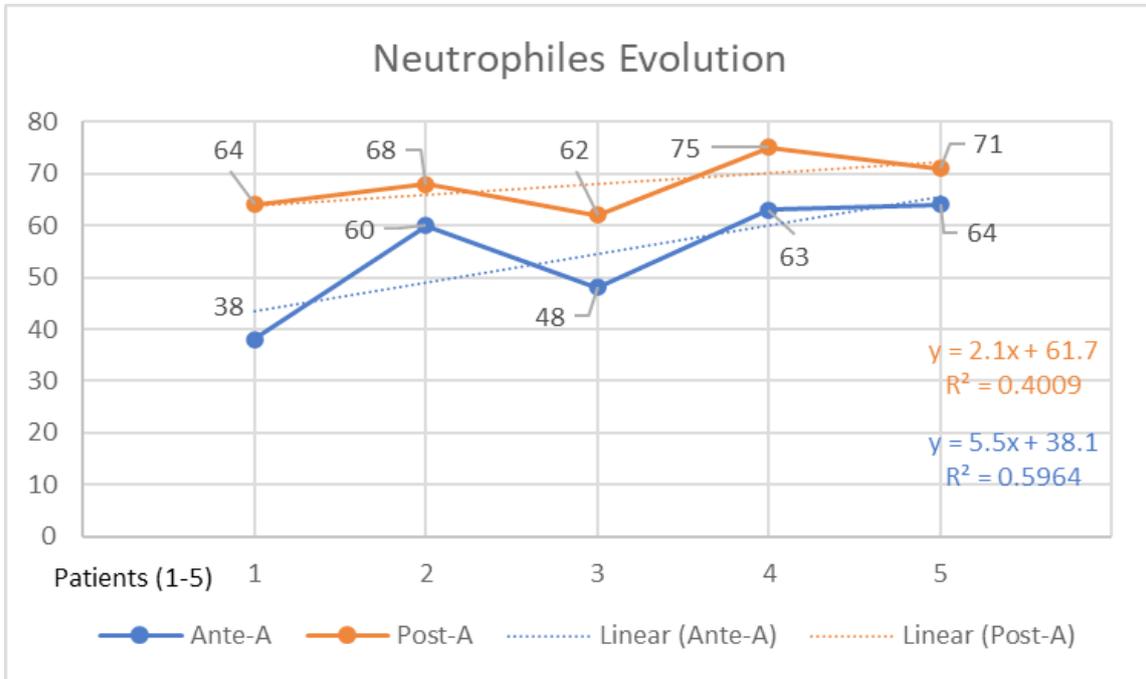


Figure 5. Neutrophils Evolution before and after the treatment

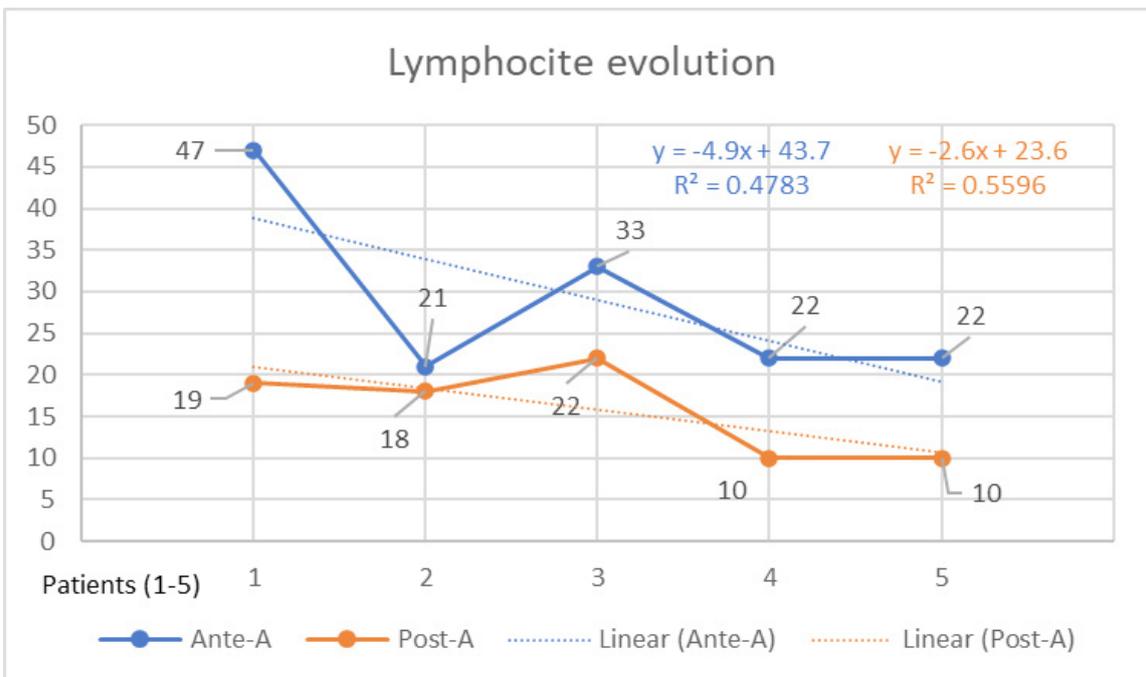


Figure 6. Lymphocyte Evolution before and after the treatment

Conclusion

The results of the present study suggest that the combination between *B. subtilis*, *B. licheniformis* and *P. acidilactici* used as a probiotic combination does not strongly influence the hematological parameters in dogs with apparent dysbiosis.

Acknowledgments. This work was supported by MICROBIOME LABS PHYSICIANS EXCLUSIVE, LLC, Agreement no 30130/18 December 2018.

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