

Natural Thermal Water Consumption Could be Capable of Improving Gut Mycobiome in Dogs with Inflammatory Bowel Disease and Dermatological Manifestations: Randomized Clinical Study

Yangısal Bağırsak Hastalığı ve Dermatolojik Bulguları Olan Köpeklerde Doğal Termal Su Tüketimi Bağırsak Mikrobiyomunu Geliştirebilir: Randomize Klinik Çalışma

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ABSTRACT Objective: Given the gut microbiome is a supreme immunological and metabolic organ, much data is recognized within the intestinal bacteria whereas the prevalence along with phylogenetic interpretation of fungal organisms participating within intestinal ecosystem have not explored convincingly among dogs with inflammatory bowel disease (IBD). In the present study, the present authors' for the first time a) described the fungal gut mycobiome, b) analyzed disbiosis network for fungal kingdom, c) detected the efficacy of natural thermal water (nTW) on gut mycobiome. **Material and Methods:** For these purpose, MIDOG All-in One Microbial Test targeting Next-Generation DNA Sequencing was used to determining of fungal microbiome prior to and thereafter nTW in a total of 13 dogs with IBD. **Results:** *Aspergillus sp.* (n=5), *Acremonium hyalinulum* (n=4), *Acremonium fusoides-hansfordii* (n=4) were frequently isolated as fungal pathogens whereas case VII exhibited 5 different species (at most) and 4 other dogs presented solely 1 species. Interestingly a 6 week course of nTW consumption resulted with disappearance of fungal pathogens in 12 out of 13 cases. On behalf of repeated Next-Generation DNA Sequencing at 6th week analysis of all 12 cases were deemed more diverse gut mycobiome. **Conclusion:** Obtained data preserved protective/restorative roles of nTW on gut mycobiome in dogs with IBD and dermatological manifestations involved at the present study.

ÖZET Amaç: Bağırsak mikrobiyomunun üstün bir immünolojik ve metabolik organ olduğu göz önüne alındığında, intestinal bakteriler için çok fazla veri tanımlanırken, yangısal bağırsak hastalığı olan köpeklerde intestinal ekosisteme katılan mantar organizmalarının filogenetik değerlendirmesi ile birlikte prevalansı ikna edici bir şekilde araştırılmamıştır. Bu çalışma ile araştırmacılar ilk defa a) fungal bağırsak mikrobiyomunun tanımlanmış, b) fungal faunadaki disbiyozis ağını tanımlanmış, c) doğal termal suyun bağırsak mikrobiyotası üzerine etkisini belirlemiştir. **Gereç ve Yöntemler:** Bu amaçlar doğrultusunda yangısal bağırsak hastalığı olan 13 köpekte doğal termal su tüketimi öncesi ve sonrası fungal mikrobiyotanın belirlenmesi için DNA sekansının belirlenmesine dayanan MIDOG ALL-in One Mikrobiyal Test kullanılmıştır. **Bulgular:** Fungal patojen olarak sıklıkla *Aspergillus sp.* (n=5), *Acremonium hyalinulum* (n=4), *Acremonium fusoides-hansfordii* (n=4) izole edilmişken, olgu VII 5 farklı tür (en fazla) ve diğer 4 köpekte sadece 1 tür tespit edilmiştir. İlginç olarak 6 haftalık termal su kullanımı sonrası 13 vakanın 12'sinde fungal patojenler kaybolmuştur. **Sonuç:** Yangısal bağırsak hastalığı ve dermatolojik bulguları olan köpeklerde doğal termal suyun bağırsak mikrobiyomu üzerindeki koruyucu/yenileyici rolü bu çalışmadan elde edilen verilerle ortaya konulmuştur.

Keywords: Alkaline water; dog; inflammatory bowel disease; gut microbiome

Anahtar Kelimeler: Alkali su; köpek; yangısal bağırsak hastalığı; bağırsak mikrobiyomu

The gastrointestinal (GI) tract anchorage a multiplex ecosystem composing several microbes such as bacteria, fungi, viruses and protozoa. This ecosystem, to our knowledge as a factory/machinery

tool, denoted ad microbiota taking into account taxonomy (“is there someone in kitchen”) and microbiome on referral to their gene ingredient and functionality (“what the hell you are doing there”).¹

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As generally known bacteria inaugurate so far the vast majority composition of these gut microorganisms, i.e. >98% of metagenomic sequencing wade through from fecal analytes denoted as bacteria in dogs and cats.² Fungal stakeholders have been recognized as a normal component of the microbiota both in small and large intestine.³ Canine inflammatory bowel disease (IBD) is a batch of disorders presented with persistent/recurrent GI issues in relationship with histopathologically proved small/large intestinal mucosal inflammation.⁴ In the vast majority of dogs presenting chronic diarrhoea and vomiting, IBD is a foremost diagnosis.⁵ Gut microbiota participate a pivotal role regarding host-microbial interactions and intestinal health. Intestinal dysbiosis might be observed/ detected in dogs with IBD which could be capable of subscribing factor.⁶ Probable alterations in gut micobiome populations in dogs with IBD must be highlighted, in which prompted us to perform this study. Figure 1 showed healthy gut and IBD gut along with intestinal fungal alterations. Someone might speculate and probably ask i) why did you perform this study? and ii) why? We do very well know that unnecessary antibiotic usage (quite common under our era of study with a great percentage, as we obtained data from the animal

owners while searching archives) should have helped unbalanced fungal communities among dogs with IBD, as we hypothesized.

Therefore we analyzed the effect of the natural thermal water (nTW) containing CO₂ and various minerals on gut mycobiome among dogs with IBD.

MATERIAL AND METHODS

For a better understanding the whole research, Table 1 was addicted to the nomenclature involved within this study.

TABLE 1: Terminological glossary for readers for better understanding the whole manuscript.	
Terminology ^{7,8}	Definition
Bacteria vs fungi	The relative abundance between bacteria and fungi
Bacteria	The percentage profile of bacterial species alone
Fungi	The percentage profile of fungi species alone
Mycobiome	-Collection of fungi -Fungal community in/on an organism ¹²
Natural thermal water ⁷	A hot/hydrothermal/geothermal spring; that is a existed by the emergence of geothermally heated groundwater onto the surface of the earth
Dysbiosis	-An unbalanced microbiota composition

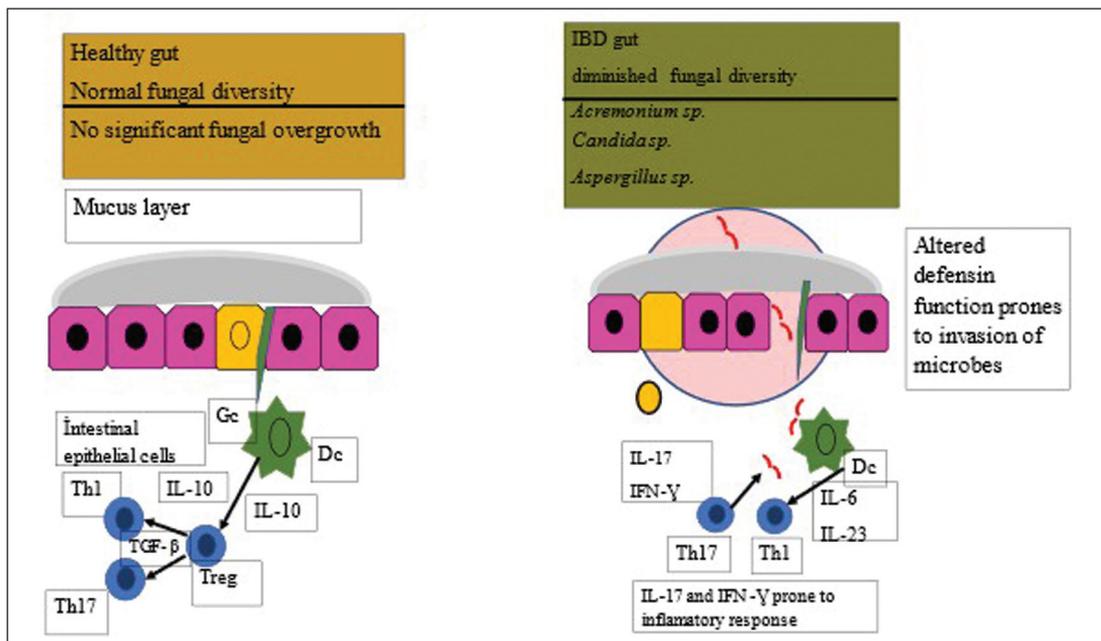


FIGURE 1: This cartoon colored representation was adopted from the original version belonging to Beheshti-Maal et al.²², and transcribed to the results of the present study herein reported.

INCLUSION CRITERIA AND DIAGNOSTIC APPROACH

Thirteen dogs, were admitted to the Aydın Adnan Menderes University, Faculty of Veterinary, Department of Internal Medicine, diagnosed with IBD were included. This was a self-controlled case series study. The minimum diagnostic interpretation evolved relevant parameters shown in [Table 1](#). Whole inclusion criteria for IBD were adapted/transformed from World Small Animal Veterinary Association similar to a description in a prior study.^{5,9} There was no prior drug application other dogs received antibiotics for at least 3 weeks prior to involvement. Diagnostic criteria, methodology and relevant analytes were shown on [Table 2](#).

SAMPLING PROCEDURE

Microbiome analysis was conducted using fecal samples that were collected 6 weeks apart [prior to and thereafter nTw application at 6th week, in which study was disrupted (end point)] throughout the study. Briefly test material obtained from Midog Center, were composed of sterile swabs, in which was used to take rectal sampling.

MYCOBIOME ANALYSIS-METHODOLOGY

The MiDOG[®] All-in-One Microbial Test (Irvine, CA, MiDOG[®] Test Center) is denoted as a targeted, Next-Generation DNA Sequencing Testing. This testing service is capable of identifying molecular signatures dedicated to the identity and character of a specific

microorganism. The latter test based on safeguarded preservation and transportation of obtained samples (by our research group), thoroughly extracted DNA (from entire microbes existed within the specimen), selected amplification of microbial DNA followed by Next-Generation DNA Sequencing [by use of the latest/novelty technologies from Illumina (Illumina, Inc., San Diego, CA)]. Data manoeuvre is performed via curated microbial databases to accurately align DNA sequences for ensuring precise and accurate (species-level) identification of all fungi present in the collected sample. Even if no fungal species were detected: relevant reasons should therefore involved very low microbial load and/or low concentration of microbial DNA within the sample sent to testing center.

ETHICAL APPROVAL

In this study was performed according to principle of Guide for the Care and Use of the Laboratory Animals and all animals rights were protected during the study procedure. And also, this study was approved by the Animal Experiments Local Ethics Committee of Aydın Adnan Menderes University numbered with 64583101/2022/79 (18/08/2022).

RESULTS

nTW (Pertek Thermal Water, Pertek, Türkiye) was the choice of natural treatment modality as because of its unchanged structure without any additives. Chemical composition of nTw was shown on [Table 3](#).

TABLE 2: Laboratory work and necessary analytes performed at the present study.

Laboratory work	Serum biochemistry, complete blood counts, faecal parasitology, urinalysis
Non-invasive imaging	Abdominal ultrasound
Inclusion criteria ⁵	I) Chronic (exceeding 21 days) persistent/recurrent gastrointestinal signs (vomiting, diarrhoea etc.) II) Exclusion of other relevant causes of gastrointestinal inflammation; III) Scant response to dietary, antibacterial (prior applications) and anthelmintic usage solely
Additional inclusion criteria developed by Feline Dermatology Group	Serum diamine oxidase levels [#] (not available histopathologic evidence of mucosal inflammation) in an attempt to detect intestinal mucosal injury/small intestinal permeability
Canine chronic enteropathy clinical activity index ¹⁰	Attitude/activity, appetite, vomiting, stool consistency, stool frequency, weight loss, albumin levels, ascites and peripheral edema, pruritus scored from 0 to 3 [which was interpreted as clinically insignificant (0-3), mild (4-5), moderate (6-8), severe (9-11), or very severe (≥ 12)] inflammatory bowel disease

[#]Canine Diamine Oxidase ELISA Kit with catalogue no: HYPERLINK "<https://www.mybiosource.com/dao-canine-elisa-kits/diamine-oxidase/739902>"MBS739902 <https://www.mybiosource.com/dao-canine-elisa-kits/diamine-oxidase/739902>

TABLE 3: Chemical composition of natural thermal water used in this study.

Natural thermal water (Pertek Thermal Water, Pertek, Türkiye) Resource: 41 °C welding heat pH=6.53		Chemical composition (selected parameters)	Unit (mg/dL)
		Calcium	366.94
		Sulphate	<0.1
		Ammonium	<0.08
		Magnesium	74.06
		Sodium	0.33
		Bicarbonate	2025
		Carbonate	1660
		Potassium	9.97

TABLE 4: Fungal mycobiome variations among diseased dogs prior to treatment. The list involved potential clinically relevant microbes detected by use of the MIDOG All-in One Microbial Test targeting Next-Generation DNA Sequencing. Different colours were used to distinguish several mycotic species obtained in each case.

Case	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
<i>Acremonium hyalinulum</i>	●				●		●						●
<i>Acremonium fusoides-hansfordii</i>	●						●						●
<i>Acremonium hansfordii</i>	●												
<i>Aspergillus sp.</i>		●		●						●	●	●	
<i>Rhizopus oryzae</i>		●											
<i>Candida tropicalis</i>			●		●			●					
<i>Aspergillus flavus</i>						●							
<i>Candida albicans</i>								●					
<i>Aspergillus subversicolor-versicolor</i>									●				
<i>Acremonium rutilum</i>							●						
<i>Hotraea werneckii</i>							●						
<i>Aspergillus candidus-tritici</i>							●						
<i>Candida sake</i>										●		●	
<i>Aspergillus flavus-minisclerotigenes-subolivaceus</i>												●	

THE MIDOG® ALL-IN-ONE MICROBIAL TEST RESULTS

MiDOG® All-in-One Microbial Test results were shown on Table 4 below. Several different fungal species were obtained, were shown prior to treatment. As was also denoted at abstracts section and thereafter discussion parts, no known pathogenic

fungal species were detected after nTw therapy of 6 weeks.

RESERVED CASE RECORDS

Case series were recorded photographically and MIDOG test reports were matched truly, as elected ones were shown on Figure 2, Figure 3, Figure 4 and Figure 5.

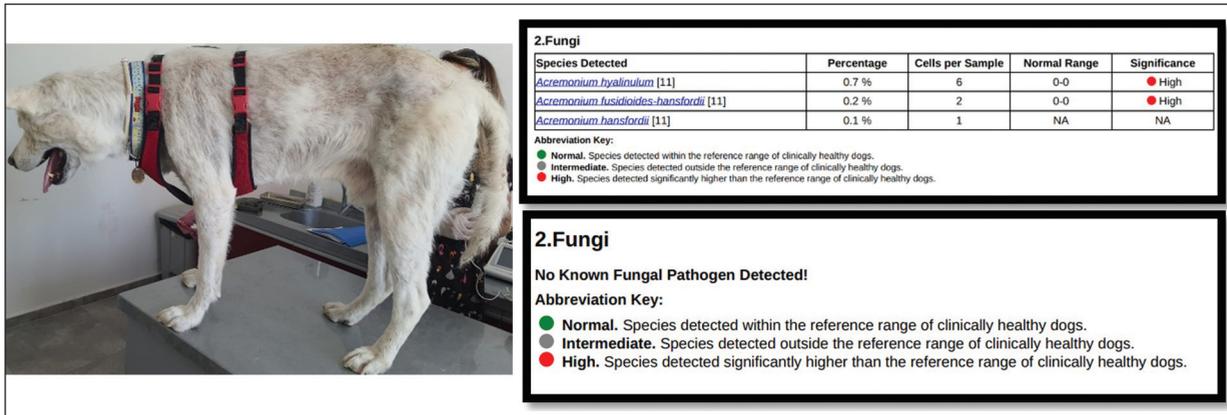


FIGURE 2: Bacterial and fungal composition of gut microbiota in case 1 prior to and thereafter thermal water application.

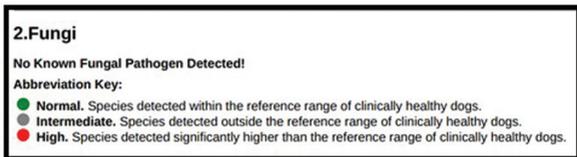


FIGURE 3: Total Fungal Composition of case IV, in which fungal species were detected, were within normal reference ranges to those of healthy dogs (according to the Midog Analysis). This was very interesting that following thermal water application for 6 weeks period, resulted with gut microbiota eubiosis regarding mycobiome in this case.

SERUM DIAMINE OXIDASE LEVELS

Serum diamine oxidase levels prior to and thereafter nTw treatment were shown in Figure 6 below. There was a significant elevation thereafter nTw treatment

indicating intestinal mucosal injury/damage and small intestinal permeability alterations.

DISCUSSION

Diverse researches among dogs and cats have exemplified that acute/chronic GI issues, involving IBD, are related to remoulding in gut microbiome communities.¹⁰ Similar imbalance regarding gut microbial community in humans with IBD might be identical to what has been observed in animals with intestinal inflammation¹¹⁻¹³ denoting that microbial respond against intestinal inflammation were preserved, in which dogs/cats might participate role model. This was one of the starting point for performing the present



FIGURE 4: Case no III with a long history of generalized alopecia within gastrointestinal issues, which were misdiagnosis. As shown Candida tropicalis was detected as fungal pathogen, which was clearly removed with nTw therapy, with no known fungal pathogene detection at week 6 after treatment.

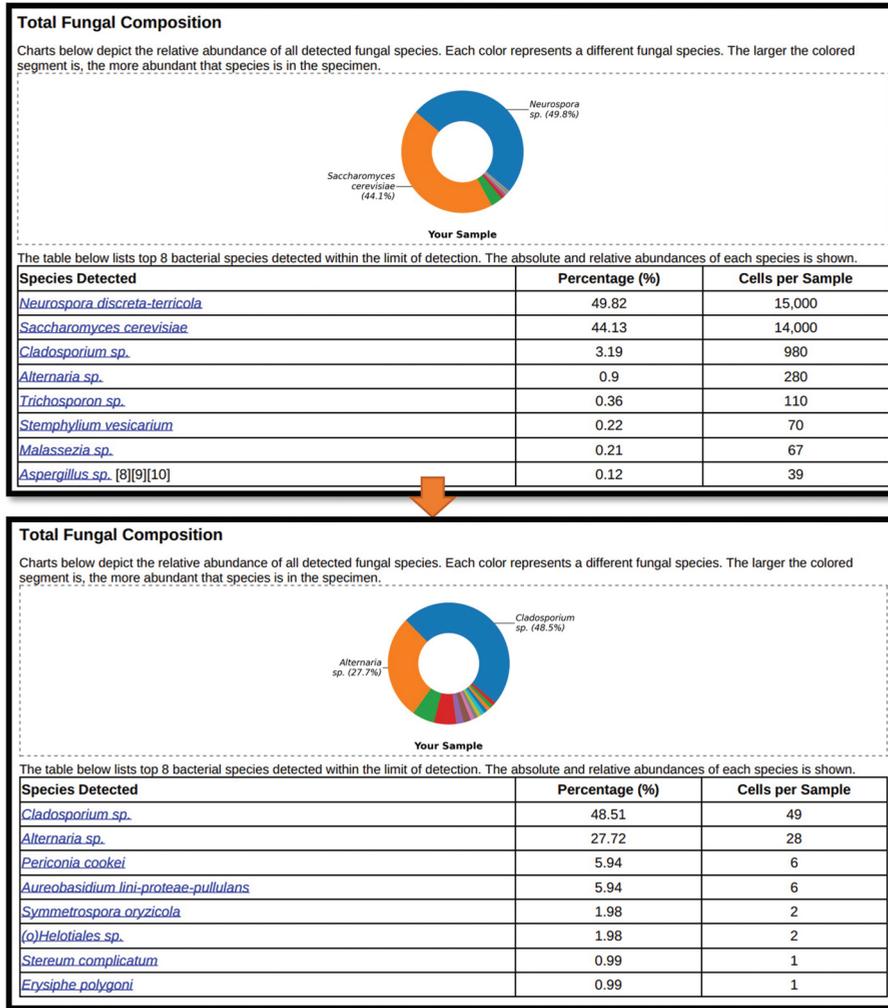


FIGURE 5: Case no XI. As seen on table I, fungal pathogens were shown in this case with iBd, whereas this figure showed out total fungal composition before and after nTw (Pertek Termal Su) application. Oral consumption of a 6 weeks period nTw resulted within absence of fungal pathogens as seen in figure at the top, which was followed by restored gut mycobiome presented in figure at bottom.

study, as available results should highlight identical disease progress in humanbeing.

A well reconized IBD, denoted as immune-mediated intestinal disorder with chronic consequences, represented by mucosal disarranging and inflammation in humanbeing.¹⁴ Given gut microbiota involves bacteria, viruses, fungi and protozoa, in which their participation among IBD cases are scant.^{15,16} Along with the gut microbiota participating a significant role (haemostasis of the immune response and other relevant ones), fungi are expressed as a lower portion of gut microbiome, indeed take place in homeostasis and mucosal

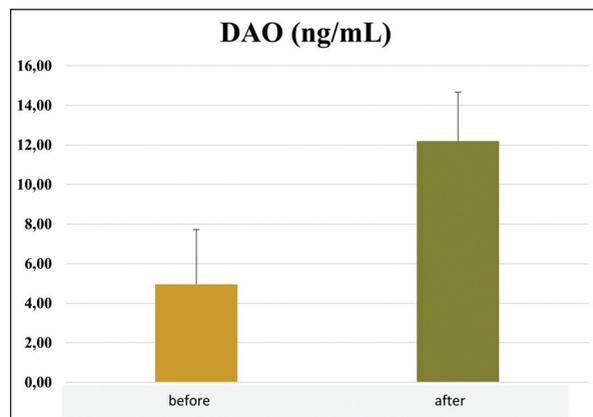


FIGURE 6: Diamine oxidase levels detected in serum prior to and thereafter nTw treatment in dogs wit iBd.

immune response.¹⁷ It has been determined that there is an interkingdom relationship among bacteria and fungi.^{17,18} However researches are lacking within the veterinary field dedicated to the role of fungi in IBD cases, which prompted us to perform this study.

In a prior research investigating oral cavity mycobiome among healthy dogs (n=12) and dogs with periodontal disease (n=39), whole maxillary arcade of each dog was sampled, in which then were forwarded to Next-Generation DNA Sequencing. A total of 320 fungal species from 135 families detected in which 3 foremost frequently species were *Cladosporium sp.* (46/51 samples), *Malassezia restricta* (44/51 samples), and *M. arunalokei* (36/51 samples). The authors concluded oral cavity of dogs with periodontal disease were presented with substantial species diversity, with several fungal communities.¹⁹ On the other hand another research aimed at detecting the prevalence and phylogenetic determination of fungal microbiome of 19 dogs (12 healthy and 7 other with acute diarrhea) by use of fungal tag-encoded FLX-Titanium amplicon (Irvine, CA, MiDOG® Test Center) pyrosequencing. According to that study 5 distinct fungal phyla were identified, with ascomycota and basidiomycota as the most abundant fungal phyla both in healthy and diseased ones. At the genus level *Candida* was the most abundant genus (both among the diseased and healthy dogs) with *C. natalensis* as identified species within the vast majority.³ In the present study if dogs participated were expressed (and therefore accepted) as diseased (prior to nTw treatment) and thereafter healthy (as gut mycobiome was taken into consideration), respectively (dysbiosis vs. symbiosis of gut mycobiome related to nTw treatment), pre-treatment analytes involved *Aspergillus sp.* (n=5), *A. hyalinulum* (n=4), *A. fusoides-hansfordii* (n=4) (were frequently isolated fungal pathogens) in a total of 13 dogs with IBD. After 6-weeks intervention of nTw therapy 12/13 dogs exhibited no known fungal pathogen as detected by Next-Generation DNA Sequencing by MiDOG analysis. This data showed us that symbiosis of gut mycobiome and restoration of microecological niche (regarding fungal species)

caused by Pertek Thermal Water nTw. It has to be claimed that 1 out of 13 cases, were unfortunately, was not suited to the protocole recommended by us, possibly interfere with the results.

Documentation obtained from gut microbiome researches and the immune respond to the GI fungi suggested a probable association between fungi and IBD. It was speculated that fungi elevated IBD risk by i) perturbation of the gut bacteria ingredient, or ii) instigating proinflammatory cytokine secretion, both of are participated within the pathophysiology of IBD. However it is still unclear which one is first “egg or hen” when taking into account the significant relationship among gut microbiota and gut mycobiome in IBD patients.¹⁷ Hence it is also not well recognized that dysbiosis of gut microbiota influenced fungal colonization or whether fungal colonization caused dysbiosis. On the other hand gut mycobiota alterations has long been well known in IBD.²⁰ In the present study gut mycobiota alterations were reversed and symbiosis of microecological niche was established by a nTw, as evidenced by restoration of gut mycobiome.

Twenty three dogs with IBD were the subject of a prior study matched with 10 healthy control dogs. The authors hypothesized whether if IBD influence gut microbial profile and host metabolism. There were no significant alterarions within the abundance of a selection of bacterial groups in contrast to control groups. In that study gut mycobiome was not analyzed, which is the subject of our study.¹¹

The results obtained in the present study might be comperable to human studies. In a prior study regarding 14 ulcerative colitis patients the prevalence of fungal taxa were analyzed. There was correlation between gut mycobiome along with pro-inflammatory cytokines in inflamed mucosa. Given that study elevated species were *Wickerhamomyces*, *Candida*, *Aspergillus* and *Sterigmatomyces*, whereas *Exophiala*, *Alternaria*, *Emericella*, *Epicoccum*, *Acremonium*, *Trametes* and *Penicillium* were decreased in comparison to healthy subjects. Contrarily elevated pro-inflammatory cytokines were not significantly related to the ascomycota/basidiomycota proportion and ascomycota to

basidiomycota ratio.²¹ Another research presented elevated *Candida* species abundance associated with mucosal inflammation of humans with IBD patients denoting a correlation between gut mycobiome shifting with inflammation and severity of disorder Crohn's disease.²² Furthermore fungal diversity among IBD cases were analyzed, in which unbalanced basidiomycota/ascomycota ratio regarding disease activity (increased basidiomycota vs decreased ascomycota in flare conditions) were determined.²³ In a prior study by use of a panfungal polymerase chain reaction, 60% of healthy dogs and 76% of dogs with chronic enteropathies showed positivity for fungal DNA within small intestinal analytes regarding the members of the phyla ascomycota (32 phylotypes) or basidiomycota (19 phylotypes). In the present study ascomycota ranged from 2.5% to 100% and were the most abundant phylum, followed by basidiomycota ranging from 2.46% to 54.62%, prior to treatment.

Given nTw composed of either carbonic metabolites/sulphates/iron presented anti-inflammatory activity (not with tap water). The rudimentary role of bicarbonates participated within vital systems involved effective GI tract protection barrier along with gut repair.²⁴⁻²⁸ In a prior and well designed research, authors evaluated the action of 2 different natural mineral water (nmw), as i) nmw I (a sulphate-carbonic-ferruginous water from the thermal springs of France) and ii) nmw II (the vast majority a bicarbonate water from the thermal springs in France), on intestinal inflammation. In that study both nmw displayed anti-inflammatory efficacy using intestinal cells. In a model of colitis induced mice, nmw I was effective in diminishing intestinal inflammation, along with modulation of the mucosa-associated microbiota.²⁶ Similarly, in our study Pertek Thermal Water, which is a nTw, exhibited close natural ingredient relationship with aforementioned nmw I (as reported by Barnich et al.), as we could speculate that intestinal inflammation was diminished.²⁹ Apart from that elevated diamine oxidase levels in response to nTw treatment in this study with Pertek Thermal Water, indicated that mucosal injury and small intestinal permeability were both reversed, in which could also contribute to the

gut mycobiome alterations observed among dogs with IBD.^{30,31}

On the other hand from a view side in efficacy of nTw used in this study must be deep analysis. Given sodium bicarbonate-NaHCO₃ (sB) usage era of i) domestic ii) medical, iii) traditional and iv) empirically, limited data regarding its activity is deemed available.³² Antifungal activity of sB on the 3 different fungal groups (yeasts, dermatophytes and molds), causative agents of skin and nail infections among humans, was the subject of an in vitro prior study. In that study antifungal activity of sB on the vast majority of integumentary fungal infection and onychomycosis (involving 70 fungal strains) was evidenced.³² Another research analyzed the efficacy of 5% sB against adherence of *C. albicans*. It was suggested that 0.12% digluconate chlorhexidine detected to present more efficacy for deduction of *C. albicans* adherence whereas 5% sB also evidenced to be a viable alternative.³³ As nTw used in this study was rich in sB, the efficacy might be dedicated to its antifungal activity.

Another interesting research (which would be also the purpose of our subsequent study, that we designed a long while ago) La Roche-Posay Thermal Spring Water (Centre Thermal La Roche-Posay, La Roche-Posay France) exhibited both probiotic and prebiotic activity promoting the diversity of the skin microbiome. The latter thermal spring water was used topically in an attempt to improve diversity of the skin microbiota along with relevant parameters (i.e. decreasing dryness and pruritus related to dermal inflammation). According to that study composition of minerals and non-pathogenic microbes in La Roche-Posay Thermal Spring Water possessed therapeutic beneficiary resulted with an increase in Gram-negative bacteria with deducing Gram-positive bacteria along with improvements in skin microbial diversity.³⁴ Our subsequent study would, thus, include nTw application in dogs (in an attempt to analyze skin mycobiota alterations).

Given probable thermal therapy of IBD patients could help control low-grade inflammation during periods of remission, with anti-inflammatory effect of nTw on colitis models, and also proof of evidence that hot spring waters might be capable of altering

gut microbiota with consumption of bicarbonate-rich mineral water, as the case in this study.^{29,35}

CONCLUSION

We herein for the first time, to our knowledge, presented that Pertek Thermal Water a nTw, preserved protective/restorative roles on gut mycobiome in dogs with IBD and dermatological manifestations involved at the present study.

Source of Finance

During this study, no financial or spiritual support was received neither from any pharmaceutical company that has a direct connection with the research subject, nor from a company that provides or produces medical instruments and materials which may negatively affect the evaluation process of this study.

Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: Kerem Ural; **Design:** Kerem Ural; **Control/Supervision:** Kerem Ural, Serdar Paşa; **Data Collection and/or Processing:** Kerem Ural, Hasan Erdoğan, Songül Erdoğan; **Analysis and/or Interpretation:** Kerem Ural; **Literature Review:** Kerem Ural, Hasan Erdoğan, Songül Erdoğan, Serdar Paşa; **Writing the Article:** Kerem Ural, Songül Erdoğan; **Critical Review:** Kerem Ural; **References and Fundings:** Kerem Ural, Hasan Erdoğan; **Materials:** Kerem Ural, Hasan Erdoğan, Songül Erdoğan, Serdar Paşa.

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