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ORIGINAL PAPER

Is there a difference in fecal microbiota of children with and without voiding dysfunction?

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Summary ***Objective:** Voiding dysfunction (VD), which encompasses many urinary symptoms that are not caused by neurological or anatomical anomalies, is a frequently encountered functional urinary bladder disorder in children. It was reported that there was an association between lower urinary tract symptoms and fecal microbiota in adult patients. Therefore, we aimed to investigate the differences in fecal microbiota between children with or without VD.*
***Methods:** Two patient groups, including 30 patients, were compared. Group 1 included patients with VD, while Group 2 consisted of healthy children. All study participants were asked to fill lower urinary tract and voiding dysfunction symptom score forms with the assistance of their parents. Subsequently, uroflowmetry tests and postvoiding residual urine measurements were performed. Fresh stool samples were collected from all children and analyzed by polymerase chain reaction. General bacterial load and presence of Roseburia intestinalis, Clostridium difficile, Fusobacterium nucleatum, and Bacteroides clarus were tested.*
***Results:** The two groups were significantly different regarding general bacterial load; the presence of Fusobacterium nucleatum. Clostridium difficile and Bacteroides clarus was not detected in the fresh stool samples of the patients in Group 2; the counts of Roseburia intestinalis were less in Group 1 than in Group 2, although there was no statistically significant difference. There was a negative correlation between symptom scores, general bacterial load, and the presence of Fusobacterium nucleatum. However, there was no correlation between the presence of Roseburia intestinalis and symptom scores.*
***Conclusions:** There is a potential relationship between VD and a deviation in the fecal microbiota in the pediatric population.*

KEY WORDS: *Voiding dysfunction; Fecal microbiota; Fecal microbiota change.*

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INTRODUCTION

Voiding dysfunction (VD) is also named bladder dysfunction in children. It is a general term encompassing both voiding and storage dysfunctions. It is a functional bladder anomaly encountered in children who do not have any neurological and anatomical abnormalities. It is not rare in children; approximately 40% of children presenting to pediatric urology clinics are affected by VD (1, 2). The human body is a complex system hosting various microorganisms, including bacteria, fungi, and parasites. The assemblage of these commensal, symbiotic or pathogenic microorganisms is called human microbiota. The human microbiota is mainly located at four anatomical regions: Skin, genitourinary system, respiratory system, and gastrointestinal system (3). The full array of these microorganisms that live on and in humans and, more specifically, the collection of microbial genomes that contribute to the genetic portrait is called the human microbiome. The specific changes in the microbiome are named dysbiosis (3). The gut microbiome plays a major role in the regulation, maturation, and function of the host immune system from the birth. The immune system has co-evolved a mutualistic relationship with the gut microbiome residing our bodies while mounting efficient responses to fight invading pathogens. Distortion of the balance between the useful and harmful intestinal microorganisms in favor of the harmful ones was associated with acute or chronic disease processes such as irritable bowel syndrome, inflammatory bowel disease, allergic diseases, obesity, depression, atherosclerosis, and colon cancer (4).

It was reported that urinary dysbiosis was associated with lower urinary tract symptoms (5). However, while the relevant studies were conducted with adult patients, none included the pediatric population. Therefore, we investigated the differences between children with and without VD regarding intestinal (fecal) microbiota.

MATERIALS AND METHODS

This study was approved by the *Ethical Review Committee of the Sitki Kocman University* (180172). Informed consent was obtained by parents or caregivers of all participants who signed the relevant forms before assignment to the study. The subjects were selected among children aged between 5 and 16 who presented to the pediatric urology and nephrology outpatient clinics. Patients with *congenital genitourinary* (GU) or *gastrointestinal* (GI) anomalies, GI diseases, acute infections, neurological anomalies, and chronic constipation were excluded. Also, patients with a history of GU surgery, GI surgery, or monosymptomatic enuresis, those treated for VD or given antibiotics, antiviral or antifungal medications during the last six months were omitted.

Any functional disturbance in voiding after the completion of toilet training was defined as VD. This occurs due to over activity or inadequate relaxation of the pelvic floor muscles, which are striated muscles under voluntary control. The

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patients presented to outpatient clinics with *lower urinary tract symptoms* (LUTS) and diagnosed with VD were included in Group 1. Group 2 consisted of healthy pediatric patients who presented to the same outpatient clinic for check-up purposes.
All study participants were evaluated regarding LUTS with the assistance of their caregivers. First, *voiding dysfunction symptom score* (VDSS) forms were filled for each subject (6). Subsequently, a uroflowmetry test was performed. Next, the voiding patterns (i.e., normal, parabolic, tower, plateau, staccato, interrupted) and voided volumes were recorded for each patient. Following this, post-voiding residual urine volumes were measured and recorded.
A 3-gram fresh stool sample was collected from all patients, and the samples were stored at -80°C.

Analysis of the stool samples

DNA isolation
Roche Magna Pure Compact robotic DNA isolation system (*Roche, Germany*) protocol was used to isolate DNA at room temperature.

DNA quantification
The DNA was quantified using a Nanodrop 2000 (*Thermo Scientific, USA*). The absorbance ratios 260/280 and 260/230 were used to assess the purity of DNA.

Real-time PCR (qPCR)
Fusobacterium nucleatum (FusN), *Clostridium difficile* (CloD), *Bacteroides clarus* (BacC), Roseburia intestinalis (RosIn) and general intestinal bacteria (16SInt) were detected in the samples.
Primers and the Taqman probe (*Hydrolysis probe*) were designed for five targets (Figure 1).
The ready-to-use lyophilized primers (5 nmol) and probes (3 nmol) were wettened on synthesis paper (*TIB Molbiol, Germany*) and diluted to 10 pmol/ul stocks.

Figure 1.
Primer sequences.

Gene	Sequencee
FusN-F	TTCAATAAAAgTggCAggTCAAg
FusN-R	TAACAACACATgCAggTCAATgg
FusN-Pr	6FAM-ACTCgAACCCCCAACCCTCggTTT--TMR
CloD-F	gCAAgTTgAgCgATTTACTTCggT
CloD-R	gTACTggCTCACCTTTgATATTYAAgAg
CloD-Pr	6FAM-TgCCTCTCAAATATATTATCCCgTATTAg--TMR
BacC-F	TCCATCCgCAAgCCTTTACT
BacC-R	gCTTCCggTgCCATTgACTA
BacC-Pr	6FAM-TTCATCATCACAgCCgACAACgCA--TMR
RosIn-F	CggATTTgCAgTggCAAgTT
RosIn-R	TgATTgCAgACgCCAATgTC
RosIn-Pr	6FAM-CgTgAAAAATCCgCgCATCTggC--TMR
16S-IntC-F	CgTCAgCTCgTgYCgTgAg
16S-IntC-R	CgTCRTCCCCRCCTTCC
16S-IntC-Pr	HEX-TTAAgTCCCRYAACgAgCgCAACCC--BBQ

LightCycler480 Probes Master (*Roche Diagnostics, Germany*) served as Enzyme&master mix. The processes were implemented in LightCycler480 II (*Roche Diagnostics, Germany*). The results were analyzed in the Abs Quant/2nd derivative analysis module. Samples creating sigmoidal curves were considered positive, while others were considered negative. General bacterial load was measured, and the presence of *Roseburia intestinalis, Clostridium difficile, Fusobacterium nucleatum*, and *Bacteroides clarus* was assessed.

Statistical analysis

The Kolmogorov-Smirnov test was used for assessing the distribution of data. Student's t-test was used to compare the groups regarding continuous variables and the chi-square test was used to compare categorical variables. The Pearson correlation coefficients (r) were used for correlation analysis. The data were displayed as means, *standard deviations* (SD), and ranges (minimum-maximum). The p value was considered statistically significant when it was less than 0,05. All statistical analyses were performed using the Statistical Package for Social Sciences software (*SPSS v24, IBM Corporation, New York, US*).

Table 1.
Demographic and clinical data and results of the comparative analysis.

Groups	Group 1	Group 2	p value
Age- year	8.26 ± 1.9	8.00 ± 1.6	0.574
Gender			0.902
Female n (%)	15 (60.0)	14 (58.3)	
Male n (%)	10 (40.0)	10 (41.7)	
Frequency (8 >)			0.001
Present n (%)	21 (84.0)	4 (16.7)	
Absent n (%)	4 (16.0)	20 (83.3)	
Urgency			0.001
Present n (%)	19 (76.0)	6 (25.0)	
Absent n (%)	6 (24.0)	18 (75.0)	
Hesitancy			0.015
Present n (%)	11 (44.0)	3 (12.5)	
Absent n (%)	14 (56.0)	21 (87.5)	
Terminal dribbling			0.001
Present n (%)	18 (72.0)	4 (16.7)	
Absent n (%)	7 (28.0)	20 (83.3)	
Low urine flow rate			0.001
Present n (%)	13 (52.0)	1 (4.2)	
Absent n (%)	12 (48.0)	23 (95.8)	
Maneuvers to hold urine			0.001
Present n (%)	19 (76.0)	2 (8.3)	
Absent n (%)	6 (24.0)	22 (91.7)	
Interrupted voiding			0.001
Var n (%)	16 (64.0)	4 (16.7)	
Yok n (%)	9 (36.0)	20 (83.3)	
Straining to void			0.001
Present n (%)	15 (60.0)	0 (0.0)	
Absent n (%)	10 (40.0)	24 (100)	
Voiding pattern			0.001
Normal n (%)	8 (32.0)	21 (87.5)	
Staccato n (%)	6 (24.0)	0 (0)	
Tower n (%)	11 (44.0)	3 (12.5)	
Plateau n (%)	0 (0)	0 (0)	
Interrupted n (%)	0 (0)	0 (0)	
Voided volume			0.001
Lower than expected bladder capacity (%)	17 (68.0)	1 (4.2)	
Consistent with the expected bladder capacity (%)	8 (32.0)	23 (95.8)	
Post-voiding residual urine volume (ml)	44.4 ± 21.6	19.4 ± 6.4	0.574
Voiding dysfunction symptom scores	21.9 ± 6.9	6.3 ± 1.3	0.001

Table 2.
The comparison of the groups regarding bacteria in the fresh stool samples.

Groups	Group 1	Group 2	p value
General bacterial load	16.5 ± 3.2	18.3 ± 2.9	0.043
Fusobacterium nucleatum	34.8 ± 2.6	37.2 ± 2.9	0.009
Clostridium difficile	33.8 ± 0	-	-
Bacteroides clarus	27.3 ± 4.4	-	-
Roseburia intestinalis	27.3 ± 4.9	28.4 ± 2.5	0.486

Table 3.
Results of the correlation analysis between voiding dysfunction symptom scores, general bacterial load and counts of specific bacteria.

	Correlation coefficient	p value
General bacterial load	-0.305	0.033
Fusobacterium nucleatum	-0.435	0.004
Roseburia intestinalis	-0.225	0.250
Bacteroides clarus	0.919	0.258
Clostridium difficile	-*	-*

** Since Clostridium Difficile was not detected in the fresh stool samples of the patients in Group 2, a correlation analysis could not be performed.*

Results

The mean patient age was 8.1 ± 0.25 (6-13). Although we planned to include 30 patients in each group, 5 patients were excluded from Group 1, and 6 patients were excluded from Group 2 due to the failure in the DNA isolation process. Thus, there were 25 patients in Group 1 and 24 patients in Group 2. Demographic data and clinical features of the study patients, including lower urinary tract symptoms, uroflowmetry, PVR measurement results, and voiding dysfunction symptom scores, are displayed in Table 1. The comparative analysis revealed that general bacterial load and the rate of *Fusobacterium nucleatum* presence were significantly lower in patients with VD than in healthy patients (p = 0.043 and p = 0.009, respectively). Although *Roseburia intestinalis* was present in fresh stool samples of both patient groups, its rate was relatively lower in the patient group with VD. *Clostridium difficile* and *Bacteroides clarus* were not detected in the fresh stool samples of the healthy patient group (Table 2). In fresh stool samples, the correlation between *voiding dysfunction symptom score* (VDSS) and general bacterial load, *Roseburia intestinalis*, *Clostridium difficile*, *Fusobacterium nucleatum*, and *Bacteroides clarus* counts were analyzed. There was a negative correlation between VDSS and general bacterial load and *Fusobacterium nucleatum* counts (p = 0.033 and p = 0.004, respectively). Although there was also a negative correlation with *Roseburia intestinalis*, it was statistically insignificant (p = 0.25) (Table 3). Since *Clostridium difficile* was not detected in the fresh stool samples of the patients in Group 2, a correlation analysis could not be performed.

Discussion

Since bowels have a 250 m^2 absorptive surface area and a nutrient-rich content, they have the most extensive flora bearing various microorganisms. Therefore, it is difficult to determine all types of bacteria and their counts included in the intestinal flora. However, investigations utilizing current methods elucidated more than 100 trillion bacteria and more than 1000 bacteria types in the bowel (7).

The microbiota, which includes various and many microorganisms, starts to develop after birth. Its initial content depends on genetic and geographical factors, route of labor, age at labor, and diet (8). continues to develop and modulate in species abundance for about 3 years, until the microbiota becomes adult-like.

Until age 1, bowel microbiota shows significantly less variation than microbiota in toddlers, adolescents, or adults. Remarkable changes occur in the content of intestinal microbiota until age 3. The primary microbiota evolves to adult microbiota after age 3 regarding the variability of bacteria types (9, 10). Anaerobic, facultative anaerobic, and aerobic bacteria are present in the gastrointestinal microbiota. Approximately 90% of this flora consists of *Bacteroides* and *Firmicutes* species. Other microbial phyla are *Actinobacteria, Proteobacteria, Verrucomicrobia*, and *Fusobacteria*. The bacteria investigated in our study were selected as per the variability in microbiota.

The association between LUTS and urinary microbiome was previously reported using 16S rRNA gene sequence (5). However, only a few studies investigated the association between intestinal microbiota and LUTS. *Holland et al.* studied 30 male patients with LUTS and suggested a significant relationship between the symptom scores and the presence of specific bacteria types in the intestinal microbiota. Of note, this study did not include a comparative analysis between patients with and without LUTS (11). *Braundmeier-Fleming et al.* compared the stool samples of the patients who had interstitial cystitis with those of healthy subjects (12). In line with our study, these researchers performed *polymerase chain reaction* (PCR) on stool samples. They reported that the counts of *E. sinensis*, *C. aerofecaciens*, *F. prausnitzii*, and *O. splanchnicus* were significantly lower in the fecal microbiota of the patients with interstitial cystitis than in healthy subjects. In a fecal microbiota study including patients with chronic prostatitis/ chronic pelvic pain syndrome (another functional lower urinary tract disorder such as interstitial cystitis) the alpha diversity analysis revealed that the diversity of fecal microbiota was significantly lower in the patient group than in healthy subjects (13). *Okamoto et al.* studied 1113 patients comparing patients with high overactive bladder symptom scores and urgency with those who had low symptom scores without urgency. They found that the former group had a significantly lower bacterial load in the fecal microbiota (14). They suggested that the natural bacterial load reduction might be correlated with the disease process. Our study determined a significant difference between patients with normal and abnormal voiding dysfunction symptom scores concerning general bacterial load and a negative correlation between VDSS and the general bacterial load. Of note, reduction in the bacterial load infers reduction of the microorganisms beneficial for health.

Some bacteria such as *Bifidobacterium* species in microbiota have beneficial critical roles, and they can be used as probiotics. These bacteria were low in patients with overactive bladder (14). On the other hand, the counts of *Faecalibacterium* species were higher in patients with overactive bladder than in the control group patients (14). Detection of high numbers of these bacteria in overactive

bladder patients is an unfavorable sign indicating the deviation in the intestinal microbiota.
In our study, *Fusobacterium nucleatum* counts were significantly lower in patients with VD than in controls (p = 0.009). The counts of *Roseburia intestinalis* were relatively lower in the former group than in the latter although the difference was not statistically significant (p = 0.486).
Clostridium difficile and *Bacteroides clarus* were not detected in the healthy patient group. Detection of these bacteria in the patient group with VD can be considered an indicator of dysbiosis. It is widely accepted that deviations in the intestinal microbiota led to an increase in the levels of toxic metabolites and a reduction in the number of useful metabolites, thus contributing to disease processes (15).
The intestine-brain axis is a two-way communication network. This network consists of the *central nervous system* (CNS), which includes the brain and the spinal cord, autonomic nervous system, enteric nervous system, and the hypothalamic-pituitary-adrenal axis (16). Thus, the intestinal microbiota can affect the enteric neurons and the CNS via metabolites secretion. A potential dysfunction affects both sides since this is a two-way interaction (17). The effects of the intestinal microbiota on brain development and the emergence of neurodegenerative diseases were also reported (18). Also, it was noted that there was a relationship between the reduction of intestinal microbial diversity and cognitive dysfunction. In addition, it was suggested that a healthy microbiota was associated with learning skills and memory development (19). Our study showed a significant reduction in the general bacterial load in the patient group with VD. Therefore, we suggest that dysbiosis could negatively affect autonomic nervous system maturation or the coordination between the CNS and the lower urinary tract.
Our study has some limitations. First, it was conducted with a limited number of patients because of *Coronavirus disease-2019* (COVID-19) pandemic during the study period. Second, the total bacterial diversity could not be analyzed since DNA sequence sampling could not be performed in fresh stool samples due to financial reasons.

Conclusions

We conclude that there is a potential relationship between VD and a deviation of the fecal microbiota. However, we further studies, including more extensive patient series, are needed in to confirm this finding.

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References

1. Farhat W, Bägli Dj, Capolicchio G, et al. The dysfunctional voiding scoring system: quantitative standardization of dysfunctional voiding symptoms in children. J Urol 2000; 164:1011-5.

2. Austin PF, Bauer SB, Bower W, et al. The standardization of terminology of lower urinary tract function in children and adolescents: Update report from the standardization committee of the International Children's Continence Society. Neurourol Urodyn 2016; 35:471-81.

3. Gill SR, Pop M, DeBoy RT, et al. Metagenomic analysis of the human distal gut microbiome. Science 2006; 312:1355-9.

4. Duvallet C, Gibbons SM, Gurry T, et al. Meta-analysis of gut microbiome studies identifies disease-specific and shared responses. Nat Commun 2017; 8:1-10.

5. Antunes-Lopes T, Vale L, Coelho AM, et al. The role of urinary microbiota in lower urinary tract dysfunction: a systematic review. Eur Urol Focus 2020; 6:361-9.

6. Akbal C, Genc Y, Burgu B, et al. Dysfunctional voiding and incontinence scoring system: quantitative evaluation of incontinence symptoms in pediatric population. J Urol 2005; 173:969-73.

7. Qin J, Li R, Raes J, Arumugam M, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature 2010; 464:59-65.

8. Pelzer E, Gomez-Arango LF, Barrett HL, Nitert MD. Maternal health and the placental microbiome. Placenta 2017; 54:30-7.

9. Arrieta M-C, Stiemsma LT, Amenyogbe N, et al. The intestinal microbiome in early life: health and disease. Front Immunol 2014; 5:427.

10. Yatsunenko T, Rey FE, Manary MJ, et al. Human gut microbiome viewed across age and geography. Nature 2012; 486:222-7.

11. Holland B, Karr M, Delfino K, et al. The effect of the urinary and faecal microbiota on lower urinary tract symptoms measured by the International Prostate Symptom Score: analysis utilising next-generation sequencing. BJU Int 2020; 125:905-10.

12. Braundmeier-Fleming A, Russell NT, Yang W, et al. Stool-based biomarkers of interstitial cystitis/bladder pain syndrome. Sci Rep 2016; 6:1-10.

13. Shoskes DA, Wang H, Polackwich AS, et al. Analysis of gut microbiome reveals significant differences between men with chronic prostatitis/chronic pelvic pain syndrome and controls. J Urol 2016; 196:435-41.

14. Okamoto T, Hatakeyama S, Imai A, et al. Altered gut microbiome associated with overactive bladder and daily urinary urgency. World J Urol 2021; 39:847-53.

15. Yin J, Liao SX, He Y, et al. Dysbiosis of gut microbiota with reduced trimethylamine-N-oxide level in patients with large-artery atherosclerotic stroke or transient ischemic attack. J Am Heart Assoc2015; 4:e002699.

16. Carabotti M, Scirocco A, Maselli MA, Severi C. Erratum: The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems. Ann Gastroenterol. 2015; 28:203-209.

17. Yang NJ, Chiu IM. Bacterial signaling to the nervous system through toxins and metabolites. J Mol Biol 2017; 429:587-605.

18. Martin CR, Osadchiy V, Kalani A, Mayer EA. The brain-gut-microbiome axis. Cell Mol Gastroenterol Hepatol. 2018; 6:133-48.

19. Davidson GL, Cooke AC, Johnson CN, Quinn JL. The gut microbiome as a driver of individual variation in cognition and functional behaviour. Philos Trans R Soc Lond B Biol Sci. 2018; 373:20170286.

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