

Microbiota analysis in individuals with type two diabetes mellitus and end-stage renal disease: A pilot study

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Received October 23, 2023; Accepted January 30, 2024

DOI: 10.3892/etm.2024.12500

Abstract. Chronic kidney disease (CKD) is a widespread health concern, which affects ~9.1% of the global population and 12-15% of individuals in upper-middle income countries. Notably, ~2% of patients with CKD progress to end-stage renal disease (ESRD), which leads to a substantial decline in the quality of life, an increased risk of mortality and significant financial burden. Patients with ESRD often still suffer from uremia and uremic syndromes, due to the accumulation of toxins between dialysis sessions and the inadequate removal of protein-bound toxins during dialysis. A number of these toxins are produced by the gut microbiota through the fermentation of dietary proteins or cholines. Furthermore, the gut microbial community serves a key role in maintaining metabolic and immune equilibrium in individuals. The present study aimed to investigate the gut microbiota patterns in individuals with type 2 diabetes mellitus (T2DM) and ESRD via quantitative PCR analysis of the 16S and 18S ribosomal RNA of selected members of the gut microbiota. Individuals affected by both T2DM and ESRD displayed distinctive features within their intestinal microbiota. Specifically, there were increased levels of Gammaproteobacteria observed in these patients, and all subjects exhibited a notably increased presence of *Enterobacteriaceae* compared with healthy individuals. This particular microbial community has established connections with the presence of inflammatory processes in the colon. Moreover, the elevated levels of *Enterobacteriaceae* may serve as an indicator of an imbalance in the intestinal microbiota, a condition known as dysbiosis. In addition, the

Betaproteobacteria phylum was significantly more prevalent in the stool samples of patients with both T2DM and ESRD when compared with the control group. In conclusion, the present pilot study focused on gut microbiome alterations in T2DM and ESRD. Understanding the relationship between dysbiosis and CKD may identify new areas of research and therapeutic interventions aimed at modulating the gut microbiota to improve the health and outcomes of individuals with CKD and ESRD.

Introduction

Recent technological advances have resulted in interest in the relationship between the microorganisms populating the intestines and human health (1). The gut microbiota contains >100 billion microorganisms, pertaining to >100 different species of bacteria, which have been proven to be involved in physiological and pathophysiological host processes. In humans, one third of the gut microbiota is common to all people, while the remaining two thirds are unique to each individual (1).

The intestinal microbiota is comprised of numerous and varied microorganisms that colonize the human intestinal tract. These have evolved alongside their human hosts for thousands of years, creating a complex relationship, with benefits for both sides (2,3). The estimated number of intestinal microorganisms (>10¹⁴) is ~10 times greater than the number of human cells, and the bacterial genome is >100 times larger than the human one (4). The microbiome has 3.3 million non-redundant genes, whereas the entire human genome only has 22,000 genes (5). The diversity of the microbiome is much greater than genomic variation; two individuals are 99.9% identical in their genomic content, but they can be 80-90% different regarding their intestinal microbiota (5).

The microbiota offers several advantages to the host organism, through its physiological actions. Some of these advantages include improving and maintaining intestinal integrity, molding the intestinal epithelium, energy production, protection against pathogens and regulation of host immunity. When the composition of the microbiota is altered, these functions are modified or lost, with various pathological implications; this state of altered microbiota is called dysbiosis (6).

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Key words: microbiota, microbiome, end-stage renal disease, type 2 diabetes, dysbiosis

Research has identified alterations of the gut microbiota specific to chronic kidney disease (CKD) and terminal/end-stage renal disease (ESRD), characterized by reduced *Roseburia*, *Akkermansia* and *Allobaculum*, and increased *Prevotella* (7). Patients with stages 3-4 of CKD present a reduction in stool aerobic microbial species, compared with clinically healthy individuals. On the other hand, patients with CKD that have not yet started dialysis treatment have been reported to exhibit an increase in aerobic microbial species in their stool samples (8). Furthermore, comparing patients with ESRD and healthy individuals, significant differences have been detected in the abundance of 190 microbial operational taxonomic units (8,9). Some microbial species that are normally present in the intestine of healthy individuals, such as *Lactobacillus* and *Prevotellaceae*, have been shown to be reduced in patients with CKD, whereas microorganisms from the *Enterobacteriaceae* genus and some species of *Enterococcus* are more abundant (8,10). In ESRD, increased aerobic species, such as *Enterococcus* and *Enterobacteriaceae*, have been observed (11). It is also known that gut dysbiosis contributes to the increase in uremic toxin concentrations in patients with CKD. This increase, in turn, favors the progression of CKD (12,13). Patients with ESRD have also been shown to exhibit reductions in butyrate-producing microbial species, including *Roseburia*, *Faecalibacterium*, *Clostridium*, *Coprococcus* and *Prevotella* (14). This category of patients has 19 microbial families that predominate; out of these 19, 12 families contain urease, 5 families contain uricase, and 3 families contain indole and p-cresyl-producing enzymes (15).

Patients on hemodialysis also have alterations in their gut microbiome. Proteobacteria, especially Gammaproteobacteria; Actinobacteria; and Firmicutes, especially the *Clostridia* phylum, have been reported to be present in increased abundance, compared with in the microbiota of healthy individuals (8).

Evidence exists of the association between alterations in intestinal microbiota and diseases such as diabetes, obesity, cancer, intestinal inflammatory disease, asthma, cardiovascular disease and renal disease (16-18). In addition, the relationship between alterations in the microbiota, diabetes mellitus (DM) and obesity has been studied intensively (19-21). Chronic low-intensity inflammatory processes have been reported to be the link between obesity and insulin resistance; however, the causal mechanism has not yet been identified (22). In addition, inflammation in adipose tissue can also impact the gut microbiota. Inflammatory signals may disrupt the delicate balance of the gut microbiome, influencing the growth and activity of specific bacterial species. This bidirectional communication between adipose tissue and the microbiota creates a complex relationship that can have significant implications for metabolic health.

The microbiota can induce weight gain in patients with diabetes or prediabetes by reducing leptin sensitivity and the cerebral expression of obesity-suppressing neuropeptides pro-glucagon and brain-derived neurotrophic factor (23). In addition, along with the host genotype, the microbiota modulates insulin secretion and diet-induced phenotypes (24) via the effect of microbial taxa on host-secreted bile acids.

In type 2 DM (T2DM), butyrate-producing bacteria are reduced, which affects the intestinal mucosa and increases gut permeability. This increased gut permeability may explain

the relationship between the microbiome, T2DM and chronic inflammation (22). Lower levels of butyrate, and other short chain fatty acids (SCFAs), determine alterations in glucose metabolism, body weight, energy production and homeostasis, and in the gut barrier function (25). An altered intestinal microbiota is a factor in the rapid progression of insulin resistance in T2DM. The underlying mechanisms include reshaping of the intestinal microbiome, and the modification of host metabolic and signaling pathways (26).

The most commonly used antidiabetic medication worldwide is metformin. This drug can affect the gut microbiome, with elevated levels of *Escherichia* spp., *Akkermansia muciniphila* and *Subdoligranulum variabile*, and lower numbers of *Intestinibacter bartlettii* detected in patients treated with metformin (27). This alteration may result in the depletion of butyrate-producing bacteria. Despite this, patients with T2DM treated with metformin have been shown to exhibit increased productions of butyrate and propionate (27).

The present study aimed to determine alterations in the intestinal microbiota of individuals with both T2DM and ESRD, to determine potential microbial mechanisms that affect the development of both diseases and to identify methods of improving the clinical state of patients within this pathological category, through intervention on the intestinal microbiome.

Materials and methods

Patients. The present study is an observational, case-control type study. Patient samples from 'NC Paulescu' National Institute of Diabetes, Nutrition and Metabolic Diseases (Bucharest, Romania) were collected between November 2019 and February 2020. A group of 9 patients with T2DM and ESRD (4 women and 5 men; mean age 61.9 years; age range, 44-80 years) were compared with a control group consisting of 8 healthy individuals (4 women and 4 men; mean age 59.5 years; age range 45-74 years). All of the participants were age matched, filled in a standardized nutritional questionnaire and signed an informed consent form. The present study was approved by the Ethics Commission of 'NC Paulescu' National Institute of Diabetes, Nutrition and Metabolic Diseases (Bucharest, Romania; approval no. Certif. 5911/04.10.2019). All participants gave their written informed consent upon inclusion in the study. The research adhered to the principles outlined in The Declaration of Helsinki and also obtained approval from the Ethics Committee at the University of Bucharest (Bucharest, Romania; under protocol code CEC reg. no. 235/9.10.2019).

T2DM was defined according to the American Diabetes Association criteria: A glycated hemoglobin level $\geq 6.5\%$ (48 mmol/mol); a fasting plasma glucose ≥ 126 mg/dl (7.0 mmol/l); a 2-h plasma glucose during 75-g oral glucose tolerance test ≥ 200 mg/dl (11.1 mmol/l); and a random plasma glucose ≥ 200 mg/dl (11.1 mmol/l) (28). CKD was defined in accordance with The Kidney Disease Improving Global Outcomes foundation guidelines as the presence of two factors: Glomerular filtration rate (GFR) < 60 ml/min and albumin > 30 mg per gram of creatinine, along with abnormalities in kidney structure or function for > 3 months. ESRD was defined as a GFR of < 15 ml/min. (29). Inclusion criteria for participants

in the present study were: Diagnosed with T2DM, >18 years of age, and diagnosed with CKD in renal replacement therapy. Exclusion criteria for participants in the study were: Non-renal disease-caused anemia; acute hemorrhaging or history of hemorrhaging in the last 3 months; blood transfusion in the last 3 months; acute inflammatory or infectious diseases; acute vascular pathology; and current immunosuppressive therapy. The patient characteristics are listed in Table I.

Microbiota analysis. Microbiota analysis was performed using culture-independent techniques, since the majority of members of the gut microbiota are not cultivatable (3,4). Bacterial DNA was extracted from stool samples using a commercial kit (Qiagen Stool Mini Kit; Qiagen, Inc.). The DNA concentrations were quantified utilizing the Qubit 4 fluorometer (Thermo Fisher Scientific, Inc.). To investigate the gut microbiota, 16S ribosomal RNA (rRNA) and 18S rRNA primers were used, and quantitative PCR was performed. The sequences of the aforementioned primers are presented in Table II. For the quantification of the various bacterial and fungal populations, the following was used: 9 ng DNA isolated from the stool samples, SYBR Green 2X (Applied Biosystems; Thermo Fisher Scientific, Inc.) and 16S/18S rRNA primers (2.5 nM). Amplification was performed on a Vii7 instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.). Each sample was analyzed in triplicate. Samples without DNA template served as negative controls. Samples were incubated at 95°C for 5 min, followed by 40 cycles at 95°C for 10 sec, 60°C for 30 sec and 72°C for 1 sec. The microbial relative quantification was performed using the 16S/18S rRNA threshold cycle values for normalization (universal 16S and 18S primers were used for normalization), and using the control group for comparison according to the $2^{-\Delta\Delta C_q}$ method (30). Species relative abundance was calculated as fold change compared to the healthy control.

Data points are presented as mean \pm SEM, and graphs were generated using GraphPad 5 software (Dotmatics). Differences between the groups were computed using the Mann-Whitney test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

There were no significant differences in the abundance of major bacterial phyla of the intestinal microbiota, Bacteroidetes and Firmicutes, between the two patient groups (Fig. 1). The intestinal microbiota of the individuals with T2DM and ESRD was characterized by increased levels of Gammaproteobacteria (Fig. 2B). In addition, all patients exhibited a significantly elevated abundance of *Enterobacteriaceae* (Fig. 2C). This microbial population is already associated with the existence of an inflammatory process in the colon, and high levels of *Enterobacteriaceae* are an indicator of intestinal dysbiosis. Additionally, the Betaproteobacteria phylum was significantly more abundant in the stool samples from the patients with T2DM and ESRD, compared with that in the control group (Fig. 2A).

The *Clostridium* genus (represented by *Clostridium leptum* and *Clostridium coccooides*) did not show any significant differences between the two groups (Fig. 3A and B). The

Table I. Characteristics of the patient group with type 2 diabetes mellitus and end-stage renal disease included in the present study.

| Characteristic | Value | |
|---------------------------------------|--------------|------------|
| | T2DM | Control |
| Sex, n (%) | | |
| Female | 4 (44) | 4 (50) |
| Male | 5 (56) | 4 (50) |
| Age, years ^a | 61.9 (3.6) | 59.5 (2.4) |
| Diabetes duration, years ^a | 16.7 (3.3) | - |
| Insulin, U/day ^a | 21.4 (8.7) | - |
| Hemoglobin, g/dl ^{a,b} | 10.4 (0.7) | 12.2 (0.6) |
| Cholesterol, mg/dl ^{a,c} | 152.2 (22.1) | 128 (15.1) |
| Triglycerides, mg/dl ^{a,d} | 192.9 (43.2) | 145 (15.0) |
| Creatinine, mg/dl ^{a,e} | 6.0 (0.8) | 0.8 (11.0) |
| Urea, mg/dl ^{a,f} | 139.3 (17.1) | 35 (10.2) |
| Calcium, mg/dl ^{a,g} | 8.8 (0.3) | 8.9 (0.4) |
| Phosphate, mg/dl ^{a,h} | 5.0 (0.4) | 2.9 (0.2) |
| Albumin, g/dl ^{a,i} | 3.9 (0.1) | 3.6 (0.1) |
| Total protein, g/dl ^{a,j} | 6.9 (0.3) | 6.6 (0.1) |

^aMean, (SEM) ^bNormal range: 11.7-15 g/dl; ^cNormal range: 125-200 mg/dl; ^dNormal range: 40-150 mg/dl; ^eNormal range: 0.5-0.9 mg/dl; ^fNormal range: 10-50 mg/dl; ^gNormal range: 8.6-10.2 mg/dl; ^hNormal range: 2.7-4.5 mg/dl; ⁱNormal range: 3.5-4.2 g/dl; ^jNormal range: 6.60-8.7 g/dl.

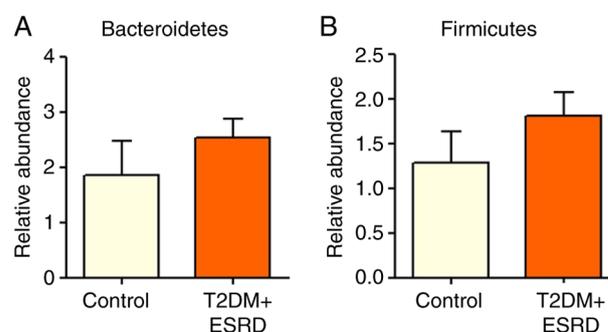


Figure 1. Relative abundance of (A) Bacteroidetes and (B) Firmicutes in patients with T2DM and ESRD. ESRD, end-stage renal disease; T2DM, type 2 diabetes mellitus.

present study also analyzed the abundance of *Butyricoccus* spp., since this is a genus with an important role in intestinal homeostasis, which has been implicated in the production of SCFAs, especially butyrate (31). Notably, the gut microbiota of individuals with T2DM and ESRD was characterized by significantly reduced levels of *Butyricoccus* (Fig. 3C).

There were no statistically significant differences in the abundance of *Ruminococcus* spp., *Faecalibacterium* spp. and *Bacteroides-Prevotella-Porphyrionas* between patients with T2DM and ESRD and healthy individuals (Fig. 4). The present study also analyzed the major fungal populations, including *Candida* spp., *Aspergillus* spp. and *Saccharomyces*

Table II. Primer sequences used for the amplification of 16S and 18S rRNA genes.

| A, Bacterial 16S rRNA | | | | |
|-------------------------|--|-----------------------------|--|---------|
| First author, year | Microbial population | Primer | Primer sequence, 5'-3' | (Refs.) |
| Yang YW, 2015 | Actinobacteria | Act664F Act941R | TGTAGCGGTGGAATGCGC AATTAAGCCACATGCTCCGCT | (56) |
| Yang YW, 2015 | Deferribacteraceae | Defer1115F Defer1265R | CTATTTCCAGTTGCTAACGG GAGATGCTTCCCTCTGATTATG | (56) |
| Yang YW, 2015 | Verrucomicrobiota | Ver1165F Ver1263R | TCAGGTCAGTATGGCCCTTAT CAGTTTTTCAGGATTTCCCTCCGCC | (56) |
| Yang YW, 2015 | Tenericutes | Ten662F Ten862R | ATGTGTAGCGGTAATAATGCGTAA CATACTTGCCTACGTACTACT | (56) |
| Yang YW, 2015 | Betaproteobacteria | Beta979F Beta1130R | AACGCGAAAAACCTTACCTACC TGCCCTTTCGTAGCAACTAGTG | (56) |
| Yang YW, 2015 | Epsilonproteobacteria | Epsilon940F Epsilon1129R | TAGGCTTGACATTGATAGAATC CTTACGAAGGCAGTCTCCCTTA | (56) |
| Yang YW, 2015 | Gammaproteobacteria | Gamma877F Gamma1066R | GCTAACGCATTAAGTACCCCG GCCATGCAGCACCTGTCT | (56) |
| Gomes-Neto JC, 2017 | <i>Mucispirillum</i> spp. | MCSP F MCSP R | TCTCTTCGGGGATGATTAAC AACTTTTCCTATATAAACATGCAC | (38) |
| DeBruyn JM, 2011 | Gemmatimonadetes | Gem F Gem R | GAATGCGTAGAGATCC CCGTCAATTCATTTGAGTTT | (57) |
| Ferreira RB, 2011 | Eubacteria: Universal primer for 16S rRNA | UniF340 UniR514 | ACTCCTACGGGAGGCAGCAGT ATTACCGCGCTGCTGGC | (58) |
| Dong Y, 2022 | <i>Lactobacillus</i> spp. | LabF362 LabR677 | AGCAGTAGGGAATTCTTCCA CACCGCTACACATGGAG | (59) |
| Rinttilä T, 2004 | BPP | F R | GGTGTGCGCTTAAGTGCCAT CGGACGTAAGGGCCGTGC | (60) |
| Matsuki T, 2004 | <i>Clostridium leptum</i> | F R | GCACAAGCAGTGGAGT CTTCCTCCGTTTTGTCAA | (44) |
| Furet JP, 2009 | <i>Clostridium coccooides</i> | F R | GACGCCGCGTGAAGG A AGCCCCAGCCTTTCACAT C | (61) |
| Noratto GD, 2014 | <i>Ruminococcus</i> spp. | F R | ACTGAGAGGTTGAACGGCCA CCTTTACACCCAGTAATTCCGGA | (62) |
| Guo X, 2008 | Firmicutes | Firm934F Firm 1060R | GGAGCATGTGGTTTAATTCGAAGCA AGCTGACGACAACCATGCAC | (63) |
| Guo X, 2008 | Bacteroidetes | Bact934F Bact1060R | GGAACATGTGGTTTAATTCGATGAT AGCTGACGACAACCATGCAG | (63) |
| Ou J, 2013 | <i>Faecalibacterium</i> spp. | F R | CCCTTCAGTGCCGCAGT GTCGCAGGATGTCAAGAC | (64) |
| Pircalabioru G, 2022 | <i>Butyricoccus</i> spp. | F R | ACCTGAAGAATAAGCTCC GATAACGCTTGCTCCCTACGT | (65) |
| Matsuki, 2004 | <i>Enterobacteriaceae</i> | Uni515F Ent826R | GTG CCA GCM GCC GCG GTAA GCC TCA AGG GCA CAA CCT CCA AG | (44) |
| B, Fungi 18 S rRNA | | | | |
| Loeffler J, 2000 | 18S rRNA universal primer | F R | ATTGGAGGGCAAGTCTGGTG CCGATCCCTAGTCGGCATAG | (66) |
| Sokol H, 2017 | <i>Saccharomyces</i> spp. | F R | AGGAGTGCGGTTCTTTG TACTTACCGAGGCAAGCTACA | (67) |
| | <i>Aspergillus</i> spp. | F R | GTGGAGTGATTTGTCTGCTTAATTG TCTAAGGGCATCACAGACCTGTT | (67) |
| Frykman PK, 2015 | <i>Candida</i> spp. | F R | TTTATCAACTTGTCACACCAGA ATCCCGCCTTACCACTACCG | (68) |

BPP, Bacteroides-Prevotella-Porphyrionas; F, forward; R, reverse; rRNA, ribosomal RNA.

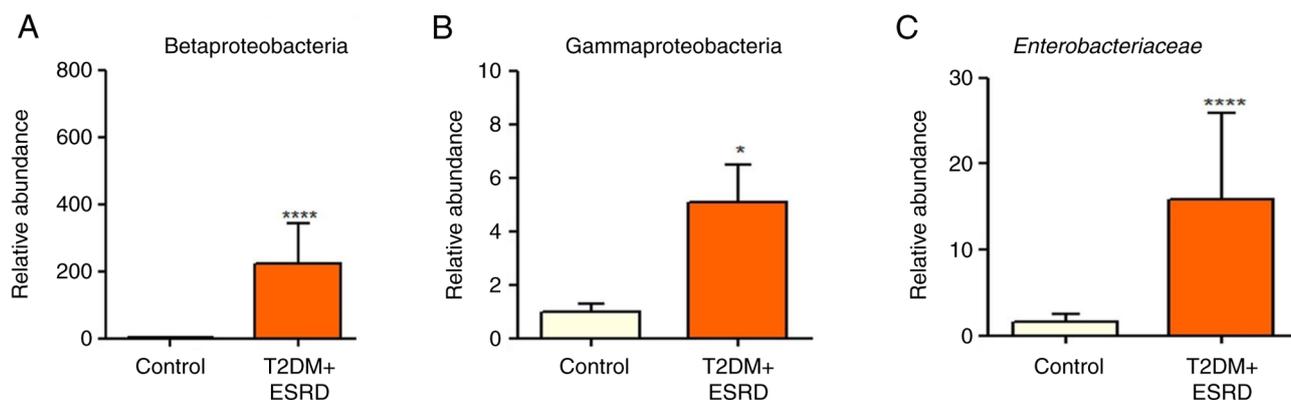


Figure 2. Relative abundance of (A) Betaproteobacteria, (B) Gammaproteobacteria and (C) *Enterobacteriaceae* in patients with T2DM and ESRD. * $P < 0.05$, **** $P < 0.0001$ vs. Control; Mann-Whitney test. ESRD, end-stage renal disease; T2DM, type 2 diabetes mellitus.

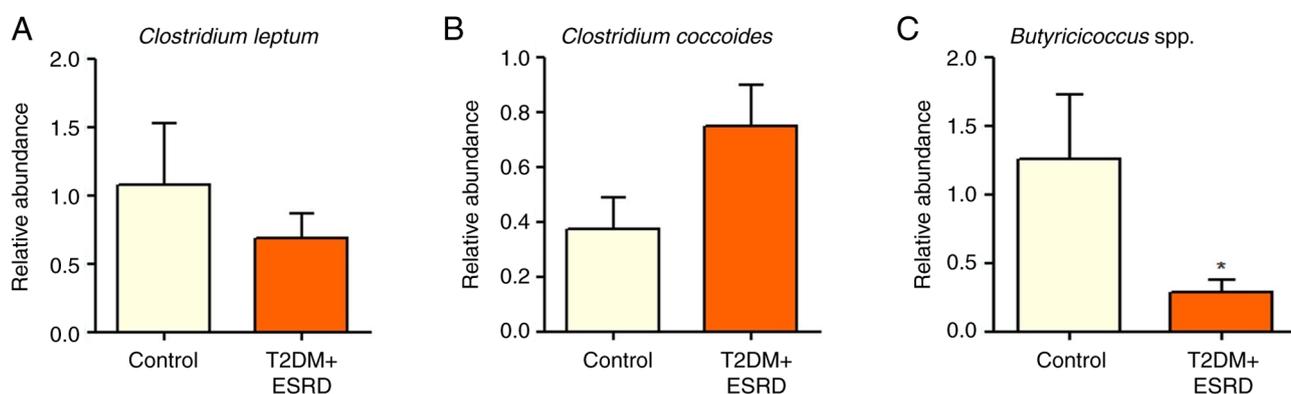


Figure 3. Relative abundance of (A) *Clostridium leptum*, (B) *Clostridium coccooides* and (C) *Butyrivococcus* in patients with T2DM and ESRD. * $P < 0.05$ vs. Control; Mann-Whitney test. ESRD, end-stage renal disease; T2DM, type 2 diabetes mellitus.

spp. While there were no statistically significant differences between the two groups regarding *Aspergillus* spp., both *Candida* spp. and *Saccharomyces* spp. were significantly increased in patients with T2DM and ESRD compared with in the control group (Fig. 5). A series of microbial phyla and populations were not identified through quantitative PCR (Cq value > 38) in the patient group studied: *Mucispirillum*, Verrucomicrobiota, Deferritbacteraceae and Tenericutes. This may be due to a very low abundance of these species in the observed individuals. In this situation, the aforementioned bacterial populations could be identified only through future advanced sequencing techniques.

Discussion

To the best of our knowledge, the present study is one of few studies that has focused on gut microbiome alterations in T2DM and ESRD (32,33). There is ample information regarding the characteristics of the intestinal microbiome in T2DM, and in CKD and ESRD, respectively; however, not much data exists regarding patients suffering from both pathologies.

Type 1 DM and T2DM are major causes of CKD, along with hypertension and glomerulonephritis; however, all mechanisms involved in the pathophysiology of CKD are not yet known. Even so, it is well known that CKD contributes to alterations in the composition and function of the gut

microbiota, resulting in dysbiosis. In addition, increasing evidence has suggested that the various toxins produced by the microbiota, including cytokines and non-self-components (i.e., uremic toxins, such as indoxyl sulfate, p-cresyl sulfate and trimethylamine-N-oxide), along with neuroendocrine molecules released in the intestines, can lead to the appearance and development of CKD. This bidirectionality is the subject of a number of recent research projects (34,35), such as the present study.

The present study on microbial DNA identified statistically significant increases in the abundance of Gammaproteobacteria, Betaproteobacteria and *Enterobacteriaceae* in the intestinal microbiota of individuals with T2DM and ESRD, compared with those in the healthy individuals in the control group. Proteobacteria is one of the most abundant intestinal phyla (36), characterized by the heterogeneity of the gram-negative bacteria that compose it. Genetic analysis of 16S rRNA allowed the division of the Proteobacteria phylum into six classes: Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria and Zetaproteobacteria (37). A number of human pathogens associated with various infectious diseases belong to this phylum: *Brucella* and *Rickettsia* in the Alphaproteobacteria class, *Bordetella* and *Neisseria* in the Betaproteobacteria class, *Escherichia*, *Shigella*, *Salmonella* and *Yersinia* in class Gammaproteobacteria, and *Helicobacter*

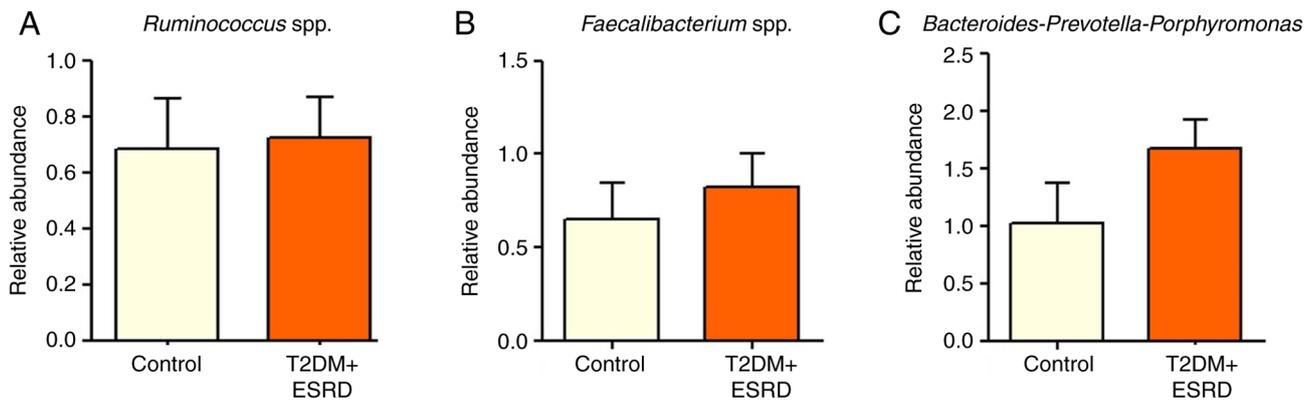


Figure 4. Relative abundance of (A) *Ruminococcus* spp., (B) *Faecalibacterium* spp. and (C) *Bacteroides-Prevotella-Porphyromonas* in patients with T2DM and ESRD. ESRD, end-stage renal disease; T2DM, type 2 diabetes mellitus.

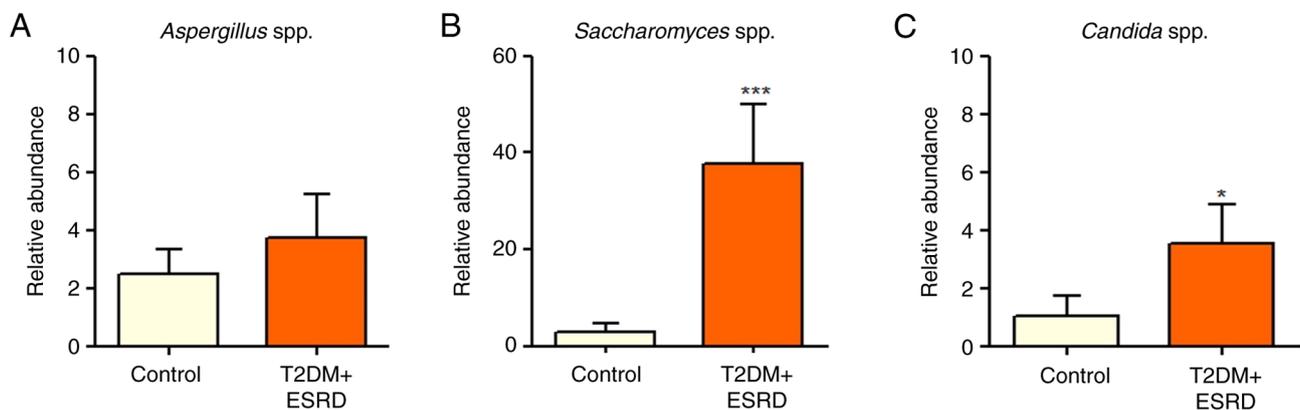


Figure 5. Relative abundance of (A) *Aspergillus* spp., (B) *Saccharomyces* spp. and (C) *Candida* spp. in patients with T2DM and ESRD. * $P < 0.05$, *** $P < 0.0001$ vs. Control; Mann-Whitney test. ESRD, end-stage renal disease; T2DM, type 2 diabetes mellitus.

in class Epsilonproteobacteria. Besides the intestines, these bacteria are also found on the skin, and in the mouth, respiratory tract, stomach and vagina (38).

Studies performed on patients with metabolic syndrome have confirmed the presence of alterations in the gut microbiota of individuals with both T2DM and prediabetes (39). Specifically, the alterations manifest through a significant increase in unknown species of the *Enterobacteriaceae* family (39). The present study confirmed these findings, implicating not only *Enterobacteriaceae*, but also Betaproteobacteria and Gammaproteobacteria. Notably, the increases in the abundance of these bacteria may result in the maintenance of a low intensity inflammatory reaction in the digestive tract and also in other parts of the body. This can be explained by the existence of anatomical (the portal venous system) and functional connections between the liver and the intestines; the gut-liver axis. The liver is the first organ confronted with high levels of lipopolysaccharide (LPS) produced by the aforementioned bacteria, which were revealed to exhibit an increased abundance in the present patient group. The K upffer macrophages recognize LPS, along with lipoteichoic acid (produced by gram-positive bacteria), as pathogen-associated molecular pattern fragments that bind to endocytosis receptors and Toll-like receptors (TLRs; e.g. TLR4 and TLR2). The result of this binding is the activation of

an inflammatory process in the hepatic parenchyma, which is accompanied by inflammatory cytokine release (IL-1 β , TNF- α , IL-6 and IL-8). These cytokines act as signaling molecules, both locally (autocrine and paracrine stimulation of K upffer macrophages, hepatocytes, endothelial cells, hepatic stellate cells and the hepatic lymphocyte population) and at a distance (stimulation of the bone marrow, hypothalamus and lymphoid structures throughout the rest of the body). The low intensity inflammatory reaction thus produced is not only present at the hepatic level, but also in the pancreas and kidneys and, in the long term, can even induce anatomical (fibrosis) and functional alterations (organ insufficiency) or cancer (40).

On the other hand, some gut microbiome metabolites, such as SCFAs (especially butyric and acetic acids), have an anti-inflammatory effect, obtained through an epigenetic mechanism involving the suppressor T cells (41). Moreover, SCFAs bind to G protein-coupled receptors and trigger various mechanisms that control obesity. These SCFAs can modulate an array of signaling pathways inside the hepatic and intestinal cells, by binding directly to transcription factors, with the purpose of maintaining homeostasis. When the gut microbiota produces excessive amounts of secondary biliary acids (deoxycholic acid and lithocholic acid), intracellular signaling becomes exaggeratedly intense and a stress response or even malignant transformation can appear (42). The present study

demonstrated that the intestinal microbiota of individuals with T2DM and ESRD was characterized by significantly reduced levels of *Butyricoccus* spp. Thus, it may be concluded that patients with this type of disease have a gut microbiota that produces significantly reduced amounts of butyric acid. This, in turn, implies a diminished anti-inflammatory effect on the intestinal and hepatic cells (42).

Intestinal dysbiosis is associated with an increased expression of genes that encode enzymes of the carbohydrate metabolic pathways. This will give bacteria an increased capacity to extract energy from the dietary products ingested by the host and will generate an excessive accumulation of adipose tissue (43). Although T2DM is mainly associated with obesity, metagenomics has also identified particularities of the fecal microbiota in patients with T2DM. Some of the studies published on this subject have identified positive correlations between improved glycemic control or insulin resistance and specific gut microbiome compositions (43,44). Research performed on Chinese patients with T2DM has shown a moderate degree of intestinal dysbiosis, with a lower abundance of butyrate-producing species and an increased number of several opportunistic classes of bacteria, such as *Enterobacteriaceae* (45).

The present study also indicated a significant increase in some fungal populations: *Candida* spp. and *Saccharomyces* spp. It is well known that candidiasis has a higher prevalence among patients with DM (both type 1 and 2). This increased abundance of *Candida* spp. in diabetes may be explained through several mechanisms, depending on the type of inflammatory reaction involved (local or systemic). Diabetes favors fungal proliferation through hyperglycemia (46), an excessive secretion of some lytic enzymes (47,48), and also through the immunosuppressive state that it creates (48,49). Some of these conditions that are favorable for fungal growth include: An easier adhesion to epithelial cells, an increased salivary level of glucose, a reduced salivary flow, microvascular degeneration and an altered neutrophil anti-*Candida* capacity (48,50). All of these factors disturb the equilibrium between the fungi and their host, and transform *Candida* from a commensal species to a pathogenic species. It has also been observed that an inadequate glycemic control increases the risk of candidiasis (51).

On the other hand, fungi from the *Saccharomyces* family are known to have beneficial effects on patients with DM, by improving blood-sugar levels, dyslipidemia, alveolar bone destruction, hepatic inflammation (as shown by a reduction in the transaminase serum levels) and by modulation of the immune response (as shown by the reduction of serum TNF- α levels) (52-54). A previous study performed on C57BL/6 mice with streptozotocin-induced diabetes demonstrated that administrating *Saccharomyces boulardii* through intraperitoneal injections during an 8-week period reduced hepatic hydroptic degeneration and hepatic vascular congestion, diminished oxidative stress (by reducing carbonylated proteins, and increasing the activity of the antioxidant enzymes superoxide dismutase and glutathione peroxidase) and normalized concentrations of the renin-angiotensin-aldosterone system peptides (affecting both the liver and the kidneys) (55).

The most interesting aspect of the present pilot study is the research population it focused on: Individuals that suffer from both T2DM and ESRD in renal replacement therapy.

Studies have already been performed that have focused on the alterations of the intestinal microbiome in both diabetes and CKD/ESRD, but there are very few, if any, studies that have focused on patients with this specific association of pathologies (32,33). Thus, the present results could shed further light upon the complex interrelations that appear between the two aforementioned pathologies and the gut microbiome. Potentially, in the future, we may offer new methods of targeting the development and progression of T2DM and ESRD, focusing on the intestinal microbiome.

One limitation of the present study is the small sample size. The healthy control group consisted of only 8 individuals, since subjects were age-matched and must have completed a nutritional questionnaire in order to avoid the impact of variables such as age and diet on the gut microbiome. In addition, when using cohorts containing older individuals, it is difficult to find matched healthy controls without other comorbidities that may also affect the gut microbiome (such as obesity, osteoporosis, cancer and autoimmune disease). More conclusive results may be obtained if the period of the study was extended and if more participants were recruited. This would allow for obtaining more significant results, and also observing the progression of the diseases and the appearance of potential complications, in relation to the changes in the gut microbiota.

In conclusion, the alterations observed in the present study suggested that local, regional (liver, pancreas and kidneys) and systemic inflammatory processes occurred in the present patient group. Because the present study consisted of patients in the advanced stages of disease, it is difficult to specify cause and effect; however, undoubtedly, gut microbiome alterations may have a role in very complex pathophysiological phenomena.

Acknowledgements

The authors would like to thank Professor Marieta Costache (Department of Biochemistry and Molecular Biology, University of Bucharest) for access to the quantitative PCR machine.

Funding

This research was funded by UEFISCDI [project ID PN-III-P1-1.1-PD-2019-0499, grant no. 224/2021 and Academia Oamenilor de Stiinta din Romania (AOSR) Teams grant no. 288/20.02.2022].

Availability of data and materials

The data generated in the present study are not publicly available due to them containing information that could compromise research participant privacy/consent but may be requested from the corresponding author.

Authors' contributions

MT was involved in drafting the manuscript, data analysis and patient recruitment. GGP was involved in study design, funding acquisition, data analysis and manuscript editing. OS was involved in data analysis, supervision, manuscript writing

and editing. MT, GGP and OS confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was conducted according to the guidelines of The Declaration of Helsinki and was approved by the Ethics Committee of University of Bucharest (protocol code CEC reg. no. 235/9.10.2019). The present study was approved by the Ethics Commission of 'NC Paulescu' National Institute of Diabetes, Nutrition and Metabolic Diseases (Bucharest, Romania; approval no. Certif. 5911/04.10.2019). All participants gave their written informed consent upon inclusion in the study.

Patient consent for publication

Patients provided written informed consent for data publication.

Competing interests

The authors declare that they have no competing interests.

References

- Luca M, Mauro MD, Mauro MD and Luca A: Gut microbiota in Alzheimer's disease, depression, and type 2 diabetes mellitus: The role of oxidative stress. *Oxid Med Cell Longev* 2019: 4730539, 2019.
- Neish AS: Microbes in gastrointestinal health and disease. *Gastroenterology* 136: 65-80, 2009.
- Bäckhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, Semenkovich CF and Gordon JI: The gut microbiota as an environmental factor that regulates fat storage. *Proc Natl Acad Sci USA* 101: 15718-15723, 2004.
- Gill SR, Pop M, DeBoy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, Gordon JI, Relman DA, Fraser-Liggett CM and Nelson KE: Metagenomic analysis of the human distal gut microbiome. *Science* 312: 1355-1359, 2006.
- Ursell LK, Metcalf JL, Parfrey LW and Knight R: Defining the human microbiome. *Nutr Rev* 70 (Suppl 1): S38-S44, 2012.
- Thursby E and Juge N: Introduction to the human gut microbiota. *Biochem J* 474: 1823-1836, 2017.
- Yang T, Richards EM, Pepine CJ and Raizada MK: The gut microbiota and the brain-gut-kidney axis in hypertension and chronic kidney disease. *Nat Rev Nephrol* 14: 442-456, 2018.
- Vaziri ND, Wong J, Pahl M, Piceno YM, Yuan J, DeSantis TZ, Ni Z, Nguyen TH and Andersen GL: Chronic kidney disease alters intestinal microbial flora. *Kidney Int* 83: 308-315, 2013.
- Ranganathan N, Friedman EA, Tam P, Rao V, Ranganathan P and Dheer R: Probiotic dietary supplementation in patients with stage 3 and 4 chronic kidney disease: A 6-month pilot scale trial in Canada. *Curr Med Res Opin* 25: 1919-1930, 2009.
- Chen YY, Chen DQ, Chen L, Liu JR, Vaziri ND, Guo Y and Zhao YY: Microbiome-metabolome reveals the contribution of gut-kidney axis on kidney disease. *J Transl Med* 17: 5, 2019.
- Hida M, Aiba Y, Sawamura S, Suzuki N, Satoh T and Koga Y: Inhibition of the accumulation of uremic toxins in the blood and their precursors in the feces after oral administration of *Lebenin*, a lactic acid bacteria preparation, to uremic patients undergoing hemodialysis. *Nephron* 74: 349-355, 1996.
- Mahmoodpoor F, Rahbar Saadat Y, Barzegari A, Ardalan M and Zununi Vahed SV: The impact of gut microbiota on kidney function and pathogenesis. *Biomed Pharmacother* 93: 412-419, 2017.
- Lau WL, Savoj J, Nakata MB and Vaziri ND: Altered microbiome in chronic kidney disease: Systemic effects of gut-derived uremic toxins. *Clin Sci (Lon)* 132: 509-522, 2018.
- Jiang S, Xie S, Lv D, Wang P, He H, Zhang T, Zhou Y, Lin Q, Zhou H, Jiang J, *et al*: Alteration of the gut microbiota in Chinese population with chronic kidney disease. *Sci Rep* 7: 2870, 2017.
- Wong J, Piceno YM, DeSantis TZ, Pahl M, Andersen GL and Vaziri ND: Expansion of urease- and uricase-containing, indole- and p-cresol-forming and contraction of short-chain fatty acid-producing intestinal microbiota in ESRD. *Am J Nephrol* 39: 230-237, 2014.
- Ramezani A and Raj DS: The gut microbiome, kidney disease, and targeted interventions. *J Am Soc Nephrol* 25: 657-670, 2014.
- Dunne JL, Triplett EW, Gevers D, Xavier R, Insel R, Danska J and Atkinson MA: The intestinal microbiome in type 1 diabetes. *Clin Exp Immunol* 177: 30-37, 2014.
- Al Khodor S and Shatat IF: Gut microbiome and kidney disease: A bidirectional relationship. *Pediatr Nephrol* 32: 921-931, 2017.
- Patterson E, Ryan PM, Cryan JF, Dinan TG, Ross RP, Fitzgerald GF and Stanton C: Gut microbiota, obesity and diabetes. *Postgrad Med J* 92: 286-300, 2016.
- Salgado MK, Oliveira LGS, Costa GN, Bianchi F and Sivieri K: Relationship between gut microbiota, probiotics, and type 2 diabetes mellitus. *Appl Microbiol Biotechnol* 103: 9229-9238, 2019.
- Xu Q, Ni JJ, Han BX, Yan SS, Wei XT, Feng GJ, Zhang H, Zhang L, Li B and Pei YF: Causal relationship between gut microbiota and autoimmune diseases: A two-sample mendelian randomization study. *Front Immunol* 12: 746998, 2022.
- Estrada V and Gonzalez N: Gut microbiota in diabetes and HIV: Inflammation is the link. *EBioMedicine* 38: 17-18, 2018.
- Schéle E, Grahnmemo L, Anesten F, Halleñ A, Backhed F and Jansson JO: The gut microbiota reduces leptin sensitivity and the expression of the obesity-suppressing neuropeptides proglucagon (Gcg) and brain-derived neurotrophic factor (Bdnf) in the central nervous system. *Endocrinology* 154: 3643-3651, 2013.
- Kreznar JH, Keller MP, Traeger LL, Rabaglia ME, Schueler KL, Stapleton DS, Zhao W, Vivas EI, Yandell BS, Broman AT, *et al*: Host genotype and gut microbiome modulate insulin secretion and diet-induced metabolic phenotypes. *Cell Rep* 18: 1739-1750, 2017.
- Wahlström A, Sayin SI, Marschall HU and Bäckhed F: Intestinal crosstalk between bile acids and microbiota and its impact on host metabolism. *Cell Metab* 24: 41-50, 2016.
- Sharma S and Tripathi P: Gut microbiome and type 2 diabetes: Where we are and where to go? *J Nutr Biochem* 63: 101-108, 2019.
- Rosario D, Benfeitas R, Bidkhorji G, Zhang C, Uhlen M, Shoaie S and Mardinoglu A: Understanding the representative gut microbiota dysbiosis in metformin-treated Type 2 diabetes patients using genome-scale metabolic modeling. *Front Physiol* 9: 775, 2018.
- American Diabetes Association: Standards of care in diabetes-2023 abridged for primary care providers. *Clin Diabetes* 41: 4-31, 2022.
- Acosta-Ochoa I, Bustamante-Munguira J, Mendiluce-Herrero A, Bustamante-Bustamante J and Coca-Rojo A: Impact on outcomes KDIGO-2012 AKI criteria according to baseline renal function. *J Clin Med* 8: 1323, 2019.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
- Takada T, Watanabe K, Makino H and Kushiro A: Reclassification of *Eubacterium desmolans* as *Butyrivibrio desmolans* comb. nov., and description of *Butyrivibrio faecihominis* sp. nov., a butyrate-producing bacterium from human faeces. *Int J Syst Evol Microbiol* 66: 4125-4131, 2016.
- Koshida T, Gohda T, Sugimoto T, Asahara T, Asao R, Ohsawa I, Gotoh H, Murakoshi M, Suzuki Y and Yamashiro Y: Gut microbiome and microbiome-derived metabolites in patients with end-stage kidney disease. *Int J Mol Sci* 24: 11456, 2023.
- Sabatino A, Regolisti G, Cosola C, Gesualdo L and Fiaccadori E: Intestinal microbiota in type 2 diabetes and chronic kidney disease. *Curr Diab Rep* 17: 16, 2017.
- Kim SM and Song IH: The clinical impact of gut microbiota in chronic kidney disease. *Korean J Intern Med* 35: 1305-1316, 2020.
- Kambay M, Onal EM, Afsar B, Dagal T, Yerlikaya A, Covic A and Vaziri ND: The crosstalk of gut microbiota and chronic kidney disease: Role of inflammation, proteinuria, hypertension, and diabetes mellitus. *Int Urol Nephrol* 50: 1453-1466, 2018.
- Rizzatti G, Lopetuso LR, Gibiino G, Binda C and Gasbarrini A: Proteobacteria: A common factor in human diseases. *Biomed Res Int* 2017: 9351507, 2017.
- Dang H, Chen R, Wang L, Shao S, Dai L, Ye Y, Guo L, Huang G and Klotz MG: Molecular characterization of putative biocorroding microbiota with a novel niche detection of Epsilon- and Zetaproteobacteria in Pacific Ocean coastal seawaters. *Environ Microbiol* 13: 3059-3074, 2011.

38. Gomes-Neto JC, Mantz S, Held K, Sinha R, Segura Munoz RR, Schmaltz R, Benson AK, Walter J and Ramer-Tait AE: A real-time PCR assay for accurate quantification of the individual members of the altered schaedler flora microbiota in gnotobiotic mice. *J Microbiol Methods* 135: 52-62, 2017.
39. Lambeth SM, Carson T, Lowe J, Ramaraj T, Leff JW, Luo L, Bell CJ and Shah VO: Composition, diversity and abundance of gut microbiome in prediabetes and type 2 diabetes. *J Diabetes Obes* 2: 1-7, 2015.
40. Ohtani N and Kawada N: Role of the gut-liver axis in liver inflammation, fibrosis, and cancer: A special focus on the gut microbiota relationship. *Hepatol Commun* 3: 456-470, 2019.
41. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, Nakanishi Y, Uetake C, Kato K, Kato T, *et al*: Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature* 504: 446-450, 2013.
42. Bäckhed F, Ley RE, Sonnenburg JL, Peterson DA and Gordon JI: Host-bacterial mutualism in the human intestine. *Science* 307: 1915-1920, 2005.
43. Gérard C and Vidal H: Impact of gut microbiota on host glycemic control. *Front Endocrinol (Lousanne)* 10: 29, 2019.
44. Matsuki T, Watanabe K, Fujimoto J, Takada T and Tanaka R: Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. *Appl Environ Microbiol* 70: 7220-7228, 2004.
45. Qin J, Li Y, Cai Z, Li S, Zhu J, Zhang F, Liang S, Zhang W, Guan Y, Shen D, *et al*: A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature* 490: 55-60, 2012.
46. Duggan S, Essig F, Hünninger K, Mokhtari Z, Bauer L, Lehnert T, Brandes S, Häder A, Jacobsen ID, Martin R, *et al*: Neutrophil activation by *Candida glabrata* but not *Candida albicans* promotes fungal uptake by monocytes. *Cell Microbiol* 17: 1259-1276, 2015.
47. Motta-Silva AC, Aleva NA, Chavasco JK, Armond MC, França JP and Pereira LJ: Erythematous oral candidiasis in patients with controlled type II diabetes mellitus and complete dentures. *Mycopathologia* 169: 215-223, 2010.
48. Rodrigues CF, Rodrigues ME and Henriques M: *Candida* sp. Infections in patients with diabetes mellitus. *J Clin Med* 8: 76, 2019.
49. Balan P, B Gogineni S, Kumari NS, Shetty V, Lakshman Rangare A, L Castelino R and Areekat K F: *Candida* carriage rate and growth characteristics of saliva in diabetes mellitus patients: A case-control study. *J Dent Res Dent Clin Dent Prospects* 9: 274-279, 2015.
50. Kadir T, Pisiriciler R, Akyüz S, Yarat A, Emekli N and Ipbüker A: Mycological and cytological examination of oral candidal carriage in diabetic patients and non-diabetic control subjects: Thorough analysis of local aetiologic and systemic factors. *J Oral Rehab* 29: 452-457, 2002.
51. Gosiewski T, Salamon D, Szopa M, Sroka A, Malecki MT and Bulanda M: Quantitative evaluation of fungi of the genus *Candida* in the feces of adult patients with type 1 and 2 diabetes-a pilot study. *Gut Pathogens* 6: 43, 2014.
52. Albuquerque RCMF, Brandão ABP, de Abreu ICME, Ferreira FG, Santos LB, Moreira LN, Taddei CR, Aimbire F and Cunha TS: *Saccharomyces boulardii* Tht 500101 changes gut microbiota and ameliorates hyperglycaemia, dyslipidaemia, and liver inflammation in streptozotocin-diabetic mice. *Beneficial Microbes* 10: 901-912, 2019.
53. Silva Vde O, Lobato RV, Andrade EF, de Macedo CG, Napimoga JT, Napimoga MH, Messoria MR, Murata RM and Pereira LJ: B-glucans (*Saccharomyces cerevisiae*) reduce glucose levels and attenuate alveolar bone loss in diabetic rats with periodontal disease. *PLoS One* 10: e0134742, 2015.
54. De Sales Guillarducci J, Marcelino BAR, König IFM, Orlando TM, Varaschin MS and Pereira LJ: Therapeutic effects of different doses of prebiotic (isolated from *Saccharomyces cerevisiae*) in comparison to n-3 supplement on glycemic control, lipid profiles and immunological response in diabetic rats. *Diabetol Metab Syndr* 12: 69, 2020.
55. Barssotti L, Abreu ICME, Brandão ABP, Albuquerque RCMF, Ferreira FG, Salgado MAC, Dias DDS, De Angelis K, Yokota R, Casarini DE, *et al*: *Saccharomyces boulardii* modulates oxidative stress and renin angiotensin system attenuating diabetes-induced liver injury in mice. *Sci Rep* 11: 9189, 2021.
56. Yang YW, Chen MK, Yang BY, Huang XJ, Zhang XR, He LQ, Zhang J and Hua ZC: Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in mouse feces. *Appl Environ Microbiol* 81: 6749-6756, 2015.
57. DeBruyn JM, Nixon LT, Fawaz MN, Johnson AM and Radosevich M: Global biogeography and quantitative seasonal dynamics of Gemmatimonadetes in soil. *Appl Environ Microbiol* 77: 6295-6300, 2011.
58. Ferreira RB, Gill N, Willing BP, Antunes LC, Russell SL, Croxen MA and Finlay BB: The intestinal microbiota plays a role in *Salmonella*-induced colitis independent of pathogen colonization. *PLoS One* 6: e20338, 2011.
59. Dong Y, Fan H, Zhang Z, Jiang F, Li M, Zhou H, Guo W, Zhang Z, Kang Z, Gui Y, *et al*: Berberine ameliorates DSS-induced intestinal mucosal barrier dysfunction through microbiota-dependence and Wnt/ β -catenin pathway. *Int J Biol Sci* 18: 1381-1397, 2022.
60. Rinttilä T, Kassinen A, Malinen E, Krogius L and Palva A: Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *J Appl Microbiol* 97: 1166-1177, 2004.
61. Furet JP, Firmesse O, Gourmelon M, Bridonneau C, Tap J, Mondot S, Doré J and Corthier G: Comparative assessment of human and farm animal faecal microbiota using real-time quantitative PCR. *FEMS Microbiol Ecol* 68: 351-362, 2009.
62. Noratto GD, Garcia-Mazcorro JF, Markel M, Martino HS, Minamoto Y, Steiner JM, Byrne D, Suchodolski JS and Mertens-Talcott SU: Carbohydrate-free peach (*Prunus persica*) and plum (*Prunus salicina*) [corrected] juice affects fecal microbial ecology in an obese animal model. *PLoS One* 9: e101723, 2014.
63. Guo X, Xia X, Tang R, Zhou J, Zhao H and Wang K: Development of a real-time PCR method for Firmicutes and Bacteroidetes in faeces and its application to quantify intestinal population of obese and lean pigs. *Lett Appl Microbiol* 47: 367-373, 2008.
64. Ou J, Carbonero F, Zoetendal EG, DeLany JP, Wang M, Newton K, Gaskins HR and O'Keefe SJ: Diet, microbiota, and microbial metabolites in colon cancer risk in rural Africans and African Americans. *Am J Clin Nutr* 98: 111-120, 2013.
65. Gradisteanu Pircalabioru G, Chifiriuc MC, Picu A, Petcu LM, Trandafir M and Savu O: Snapshot into the type-2-diabetes-associated microbiome of a Romanian cohort. *Int J Mol Sci* 23: 15023, 2022.
66. Loeffler J, Hebart H, Magga S, Schmidt D, Klingspor L, Tollemar J, Schumacher U and Einsele H: Identification of rare *Candida* species and other yeasts by polymerase chain reaction and slot blot hybridization. *Diagn Microb Infect Dis* 38: 207-212, 2000.
67. Sokol H, Leducq V, Aschard H, Pham HP, Jegou S, Landman C, Cohen D, Liguori G, Bourrier A and Nion-Larmurier I: Fungal microbiota dysbiosis in IBD. *Gut* 66: 1039-1048, 2017.
68. Frykman PK, Nordenskjöld A, Kawaguchi A, Hui TT, Granström AL, Cheng Z, Tang J, Underhill DM, Iliev I, Funari VA, *et al*: Characterization of bacterial and fungal microbiome in children with hirschsprung disease with and without a history of enterocolitis: A multicenter study. *PLoS One* 10: e0124172, 2015.

