Differences in faecal microbiome composition between adult patients with UCD and PKU and healthy control subjects

C. Timmer a,*, M. Davids b, M. Nieuwdorp b, J.H.M. Levels b, J.G. Langendonk c, M. Brederveeld c, N. Ahmadi Mozafari c, M. Langeveld a

a Department of Dietetics and Nutritional science and Department of Endocrinology and Metabolism, Amsterdam University Medical Centers, Amsterdam, the Netherlands
b Department of Vascular Medicine, Amsterdam University Medical Centers, Amsterdam, the Netherlands
c Department of Dietetics and Department of Internal Medicine, Center of Lysosomal and Metabolic Diseases, Erasmus University Medical Center, Erasmus MC, Rotterdam, the Netherlands

ARTICLE INFO

Keywords:
Microbiome
Gut
Faecal
Urea cycle defect
Phenylketonuria
Hyperammonemia

ABSTRACT

Urea cycle disorders (UCDs) are a group of rare inherited metabolic diseases causing hyperammonemic encephalopathy. Despite intensive dietary and pharmacological therapy, outcome is poor in a subset of UCD patients. Reducing ammonia production by changing faecal microbiome in UCD is an attractive treatment approach. We compared faecal microbiome composition of 10 UCD patients, 10 healthy control subjects and 10 phenylketonuria (PKU) patients. PKU patients on a low protein diet were included to differentiate between the effect of a low protein diet and the UCD itself on microbial composition. Participants were asked to collect a faecal sample and to fill out a 24 h dietary journal. DNA was extracted from faecal material, taxonomy was assigned and microbiome data was analyzed, with a focus on microbiota involved in ammonia metabolism.

In this study we show an altered faecal microbiome in UCD patients, different from both PKU and healthy controls. UCD patients on dietary and pharmacological treatment had a less diverse faecal microbiome, and the faecal microbiome of PKU patients on a protein restricted diet with amino acid supplementation showed reduced richness compared to healthy adults without a specific diet. The differences in the microbiome composition of UCD patients compared to healthy controls were in part related to lactulose use. Other genomic process encodings involved in ammonia metabolism, did not seem to differ. Since manipulation of the microbiome is possible, this could be a potential treatment modality. We propose as a first next step, to study the impact of these faecal microbiome alterations on metabolic stability.

Take home message: The faecal microbiome of UCD patients was less diverse compared to PKU patients and even more compared to healthy controls.

Abbreviations: 16S rRNA, taxonomic marker genes, common to all bacteria; ADI, Arginine Deimination. Bacteria derive energy from the deamination of arginine to citrulline and citrulline cleavage to ornithine plus carbamoyl phosphate. The latter is then converted into ATP and carbon dioxide, or used for pyrimidine biosynthesis. This route also generates two moles of ammonia (one from the arginine-citrulline conversion, the second from carbamoyl phosphate hydrolysis); Alpha Diversity, the species diversity in a microbial sample. Used to represent the taxonomic diversities of individual samples; Ammonium scavengers, agents developed for the reduction of blood ammonia concentration used for the treatment of patients with urea cycle disorders. Sodiumbenzoate and phenylbutyrate are ammonium scavengers; ASLΔ, argininosuccinate lyase (ASL) deficiency; ASSD, argininosuccinate synthetase (ASS) deficiency; ASV, Amplified Sequence Variant. A specific nucleotide sequence representing a bacterial lineage; ARG1Δ, arginase 1 (ARG1) deficiency; BCAA, branched chain amino acids: isoleucine, leucine and valine; DEGs, differentially expressed genes; DESeq, an R package to analyse count data from high-throughput sequencing assays such as RNA-Seq and test for differential expression; EAA supplement, essential amino acids supplement containing L-histidine, L-isoleucine, L-leucine and valine; DEGs, differentially expressed genes; DESeq, an R package to analyse count data from high-throughput sequencing assays such as RNA-Seq and test for differential expression; EAA supplement, essential amino acids supplement containing L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-threonine, L-tryptophan and L-valine with optional L-cystine and L-tyrosine added (depending on what product is used); FPD, Faiths Phylogenetic Diversity, alpha diversity metric accounting for genetic diversity; Genus, a taxonomic rank; Metagenome, microbiome collective genome; OTCG, ornithine transcarbamylase deficiency; PFAA, precursor free amino acid supplement, in this case phenylalanine free; PKU, Phenylketonuria; PCoA, Principal Coordinate Analysis. PCoA is aimed at graphically representing a resemblance matrix between p elements (individuals, variables, objects, among others). By using PCoA we can visualize individual and/or group differences. Individual differences can be used to show outliers; Proteolytic capacity, the capacity to break proteins down into smaller polypeptides or amino acids. In this study: enzymes involved in protein degradation; RT-qPCR, real-time quantitative polymerase chain reaction; Sodium BPA, sodium phenylbutyrate; UCD, urea cycle defect.

* Corresponding author.
E-mail address: c.timmer@amsterdamumc.nl (C. Timmer).

https://doi.org/10.1016/j.ymgmr.2021.100794
Received 10 August 2021; Accepted 19 August 2021
Available online 8 September 2021
2214-4269/© 2021 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
1. Introduction

Urea cycle disorders (UCDs) are a group of rare inherited metabolic diseases causing hyperammonemic encephalopathy. Treatment of patients with UCDs consists of reducing nitrogen load (protein-restricted diet), improving residual urea cycle function (arginine and/or citrulline supplementation), removal of nitrogen by using alternative pathways (with drugs like sodiumbenzoate and/or phenylbutyrate) [1] and/or decreasing the intestinal production of ammonia and/or its absorption into the body (administration of antibiotics or lactulose) [2]. If natural protein tolerance is lower than the FAO/WHO/UNU 2007 safe levels of protein intake (0.83 g natural protein per kilogram bodyweight for adults), supplementation of essential amino acids (EAA) is given to ensure adequate amino acid availability for growth and maintenance [1].

Despite intensive dietary and pharmacological therapy, outcome is poor in subset of UCD patients [1,3], with frequent hyperammonemic decompensations and impaired physical functioning and poor quality of life. In these patients liver transplantation could be considered as a last resort [4,5].

Under normal conditions, gut bacteria produce a significant part of ammonia circulating in the body [6], with predictions varying between 20 and > 50% [7]. In healthy individuals, the composition of the faecal microbiome can be significantly influenced by diet [8,9]. In the first years of life, the diversity of the faecal microbiome increases. During adult life, the core composition remains relatively constant [8]. UCD patients are treated with a protein restricted diet that greatly differs from a healthy diet of ‘normal’ diet. To ensure sufficient caloric intake, UCD patients eat more carbohydrates, and essential amino acids are supplemented. All these elements separately can change the faecal microbiome composition [10–16]. Higher carbohydrate intake can reduce faecal microbiome diversity [17]. In vitro growth of human intestinal bacteria on a mixture of amino acids results in enrichment of pathogenic species such as Escherichia Coli and Shigella [12]. These bacteria are known to produce ammonia [13]. In the colon, ammonia can be generated by microbial fermentation of glutamine, serine, threonine, and glycine [14]. In addition, the use of l-carnitine, as well as lactulose (regularly used by UCD patients and not by PKU patients) can have an effect on faecal microbiome composition. Overall, changes in microbiome composition in UCD patients can be expected, and compositional and functional shifts of the microbiome towards ammonia production can potentially have a negative effect on metabolic control.

From a treatment perspective, reducing ammonia production by faecal microbiome as a way of improving metabolic control in UCD, is an attractive approach [2]. In a mouse model of hepatic injury, introduction of an engineered microbiota with reduced urease activity decreased gut ammonia production, improved morbidity and reduced mortality [18]. Administration of probiotics may have a positive effect on clinical manifestations in patients with hepatic encephalopathy, though well designed trials of sufficient size are lacking [19]. In UCD patients, currently a phase II trial is conducted studying the effect of a low protein diet (less than 0.83 g protein/kg/day) and use of amino-acid supplementation. All these elements separately can change the faecal microbiome composition [18].

Hypothesis: In urea cycle defect patients, the protein restricted diet in combination with essential amino acid supplementation, results in a different faecal microbiome composition compared with healthy individuals without a specific diet.

1.1. Aims

- To detect differences between faecal microbiome composition of UCD patients, PKU patients and healthy controls.
- To study the relative abundance of ammonia producing bacterial species in the microbiome of UCD patients versus healthy controls and UCD patients versus PKU patients.
- To study the influence of UCD itself versus the protein restriction and amino acid supplementation on microbiome composition by comparing UCD and PKU patient outcomes.

2. Methods

Since no previous studies on microbiome composition in UCD patients have been carried out, power calculation was based on a study looking at differences in the abundance of bacterial species in patients with liver cirrhosis with encephalopathy versus healthy control subjects [18]. To pick up the reported 7.3% difference in relative abundance of Firmicutes Clostridiales (known to produce ammonia) we need a sample size of at least 6 subjects per group (2-sided chi-square test with a desired alpha of 0.05 and a desired power of 0.8). To be able to pick up differences in other bacterial strains as well, we included as many patients possible (estimated were 15 eligible UCD patients at the Erasmus and Amsterdam Medical Centers combined). Between 2017 and 2019 adult UCD and PKU patients, attending the outpatient clinic for inherited metabolic diseases of Erasmus MC and Amsterdam UMC, location AMC, were asked to participate. Inclusion criteria were a definite diagnosis of classic PKU or UCD, treatment with a low protein diet (less than 0.83 g protein/kg/day) and use of amino-acid supplementation. Exclusion criterion was antibiotics use in the 3 months preceding inclusion. From 3 UCD patients a second stool sample was collected at hospital admission for hyperammonemic encephalopathy. Healthy adults were recruited via advertisements in 2018–2019. These healthy controls were unrelated to the patients, and not sharing a household. The inclusion criteria were: age over 18 years and the ability to give informed consent. Exclusion criteria were a specified diet and antibiotics, probiotics or laxatives intake or other medication likely to influence gut transit time in the 3 months preceding inclusion. All participants, or their legal representative, gave written informed consent. The study was approved by the regional ethics committees (Amsterdam UMC, location AMC and Erasmus MC) and followed the
stored at frozen cooling element and sent to AMC until essay. All samples were collected by participants. Faecal samples were cooled by a 1 day dietary record. The first faecal sample on the next working day was collected by participants. Faecal microbiome data was analyzed and visualized in R (V3.6.3). Principal coordinate analysis of the microbiome Bray-Curtis dissimilarity shows almost complete separation of the UCD group and the other 2 groups, and grouping accounts for 15% of the observed variance between the 3 groups (Fig. 1B; permanova; p = 0.001; R2 = 0.15). Most UCD patients (n = 6; 67%) used laxatives (33% Lactulose, 22% Macrogol, and 11% both), and one of the 10 PKU patients (10%) in our cohort used Macrogol. Laxative use was the main driver of the observed differences in microbiome composition between UCD patients, PKU patients and healthy controls (p = 0.003; R2 = 0.10). Other parameters that had an association with faecal microbiome composition were the use of arginine and carnitine supplementation (p = 0.066) (Supplementary Fig. 4). After correcting for laxative use, microbiome composition was still statistically significant different between UCD patients versus both PKU patients and controls (p = 0.015; R2 = 0.1).

Several taxa showed significant differential abundance between healthy control subjects and UCD patients and between healthy control subjects and PKU patients (Fig. 2).

Compared to control subjects, the microbiome of UCD patients shows a reduced abundance of strictly anaerobic Clostridia and increased abundance of facultative anaerobic clades, characteristic for the microbiome of the upper GI tract. Differences between PKU and control subjects is characterized by changes in the order of Phascolarctobacteriales. The functional characteristics of the microbiota involved in ammonia metabolism are more prevalent in the microbiome of PKU subjects compared to the microbiome of healthy control subjects, the Ruminooccusaceae are less prevalent compared to the microbiome of healthy control subjects.

3.3. Microbiome ammonia metabolism

The functional characteristics of the microbiota involved in ammonia metabolism are displayed in Fig. 3.
Overall the results showed a decrease in proteolytic capacity (enzymes involved in protein degradation) in UCD patients compared to healthy controls, that may be even more pronounced during hyperammonemic decompensation. Ammonia-lyase and amino acid oxidoreductase potential do not differ. Urease potential (EC:3.5.1.5) also did not differ between the three groups. Glutamine synthase (GS: EC:6.3.1.2) and carbamoyl-phosphate synthase (CPS: EC: 6.3.5.5) showed no differences between the three groups.

The total protein intake (natural protein plus disease specific L-amino acid based protein substitutes) of UCD patients was on average 51% of the protein intake of healthy controls, and 53% of the total protein intake of PKU patients. The total protein intake of PKU patients (natural protein and PFAA) was 95% of healthy controls and therefor in the normal range.

### 3.4. Faecal short chain fatty acids (SCFA)

There was no statistically significant difference in faecal SCFA composition between the three subgroups (UCD, PKU, CON).

### 4. Discussion

The microbiome of UCD patients is significantly different when compared to PKU patients and healthy control subjects. The obligate treatment for both UCD and PKU patients is a low natural protein diet, complemented with amino acid supplementation for sufficient protein...
intake. Laxatives are frequently used in the UCD group and are well-known to change microbiome composition [2,13]. The major difference in microbiome content between the studied groups is associated with lactulose use, which was only used by UCD patients. The faecal microbiome of those UCD patients contain an increased abundance of facultative anaerobic clades, known to be associated with a short intestinal transit time. As the use of arginine and carnitine supplementation was associated with differences in faecal microbiome as well as laxative use, the observed difference in microbiome composition may be due to collinearity. As reported previously [16,23,28], the faecal microbiome of PKU patients differs from that of healthy control subjects. In this study we show for the first time a different faecal microbiome in UCD patients, compared to both PKU and healthy controls. Healthy control subjects have the most rich and diverse faecal microbiome of the 3 groups, while PKU patients showed a reduced richness and UCD patients a less diverse microbiome. A less rich and/or diverse microbiome is associated with different health issues, such as low-grade inflammation, obesity and metabolic syndrome [17,29,30]. Whether the altered faecal microbiome diversity affects the health or whether it is a disease marker is still unknown. The impact of these presented findings on morbidity and complications in UCD and PKU patients cannot be given based on the presented findings, this requires a prospective and larger study.

In healthy adults, bacteria belonging to the families Lachnospiraceae and Ruminococcaceae co-dominate the faecal bacteria [31]. Ruminococcaceae are known butyrate producers, while Lachnospiraceae are mainly propionate producers. Butyrate and propionate are two of the main short chain fatty acids (SCFA) metabolites. We found a higher prevalence of several members of Lachnospiraceae in the faecal microbiome of PKU patients, whilst multiple lineages of Ruminococcaceae species were reduced. The increased abundance of Lachnospiraceae was also seen in the faecal microbiome of pigs fed with a comparable diet to PKU patients: a low natural protein diet supplemented with amino acids [32]. Two studies with PKU patients reported opposite findings: both reported reduced prevalence of Lachnospiraceae [16,28]. In the study of Pinheiro de Oliveira [16] some patients used antibiotics (25% of the PKU group and 30% of the CON group used antibiotics in the 6 months preceding the faecal sample collection) and all patients were children. Antibiotics might explain the contrasting findings. Also the microbiome of children is different from adults [33]. The study by Mancilla [28] doesn’t report antibiotic or other medication use. Both our study and these two studies were performed in small groups, with 8–10 patients per group, which can result in type 2 errors. The large intra individual variations in specific groups of bacteria can also result in type 2 errors.

The inferred genomic capacity of the faecal microbiome composition shows a selection for a less proteolytic microbiota in UCD patients. This was not observed in PKU patients, suggesting that the total protein intake (natural protein plus PFAA) may be used as a protein source by
Relati
relative abundance
0.0325
0.0350
0.0400
0.0425
0.0450
2e−04
4e−04
6e−04
contribute to gut ammonia production. Changes in mucus microbiome
protective layer. Glycoproteins are metabolized by gut microbes and
homeostasis may thus contribute to metabolic stability in UCD patients.
These glycoproteins are secreted into the gut, where they serve as a
tract [7]. Another contributing factor besides urea degradation is the use
of systemic glutamine. We hypothesize that a significant amount of
ammoniacal decompensation. Determining ammonia-lyases and amino
acid oxidoreductase activity will be a better proxy for amino-acid
fermentation then the PICRUSt2 inferred genomic blue print as a po
factors 
5. Conclusion
Adult UCD patients and PKU patients, both on a low protein diet with
amino acid supplementation, have a different faecal microbiome
composition compared to healthy controls without a specific diet.
Healthy control subjects have the most rich and diverse microbiome of
the 3 groups. The differences in the faecal microbiome composition of UCD patients compared to healthy controls are in large part explained by lactulose use. Whether the microbiome alterations influence metabolic stability in UCD patients, and whether manipulation of the microbiome is a potential treatment modality, should be determined in future studies.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymgmr.2021.100794.

Funding

This study was supported by ESN, the Dutch Society for Inborn Errors of Metabolism. M.N. is supported by a personal ZONMW VICI grant 2020 [09150182010020].

Data availability

Raw sequence reads were submitted to the European Nucleotide Archive (ENA) and can be found under study PRJEB41032.

Author contributions

CT and ML participated in the planning and conducting of the project. CT, JGL, NAM and MB participated in acquisition of data. JHML analyzed and reported the SCFA data. CT wrote the first draft of the project. CT, JGL, NAM and MB participated in acquisition of data. MD analyzed and reported the microbiome data and provided the figures. JHML analyzed and reported the SCFA data. CT wrote the first draft of the manuscript and provided the final approval for the submission. All authors have participated in drafting the manuscript or revising it critically for important intellectual content. All authors provided approval for the submission.

Declaration of Competing Interest

CT, MD, JHML, JGL, MB and NAM declare to have no conflict of interest. ML is involved in premarketing studies with Genzyme, Protalix and Idorsia. MN is in the Scientific Advisory Board of Caelus Pharmaceuticals, the Netherlands and Kaleido, USA. None of these are directly relevant to the current paper.

Acknowledgments

We thank Laura van Dussen for her help with the ethical committee approval process, and Hilde Herrema and Jorn Hartman for their assistance and sequencing work at the Microbiota Center Amsterdam. The authors also thank the participants for contributing stool samples and keeping a dietary record, which enabled us to conduct this study.

References

[1] J. Haberle, A. Burlina, A. Chakrapani, et al., Suggested guidelines for the diagnosis and management of urea cycle disorders: first revision, J. Inherit. Metab. Dis. 42 (2019) 1192–1230.
[2] J. Liu, E. Li, Qiaoyan, H.J. Chung, H.J. Kim, S.T. Hong, The pharmacological approach to treat hyperammonemia, Nutrients 10 (2018).
[3] R. Posset, A.I. Groppman, S.C.S. Nagamani, et al., Impact of diagnosis and therapy on cognitive function in urea cycle disorders, Ann. Neurol. 86 (2019) 116–128.
[4] S. Kolker, V. Valayamopoulou, A.B. Burlina, et al., The phenotypic spectrum of organic acidurias and urea cycle disorders, part 2: the evolving clinical phenotype, J. Inherit. Metab. Dis. 38 (2015) 1059–1074.
[5] V. Walker, Ammonia toxicity and its prevention in inherited defects of the urea cycle, Diabetes. Obes. Metab. 11 (2009) 823–835.
[6] S. Williams, Review article: bacterial flora and pathogenesis in hepatic encephalopathy, Aliment. Pharmacol. Ther. 25 (Suppl 1) (2007) 17–22.
[7] D.G. Levitt, M.D. Levitt, A model of blood-ammonia homeostasis based on a quantitative analysis of nitrogen metabolism in the multiple organs involved in the production, catabolism, and excretion of ammonia in humans, Clin. Exp. Gastroenterol. 11 (2018) 193–215.
[8] G.D. Wu, J. Chen, C. Hoffmann, et al., Linking long-term dietary patterns with gut microbial enterotypes, Science 334 (2011) 105–108.
[9] L.A. David, C.F. Maurine, R.N. Carmody, et al., Diet rapidly and reproducibly alters the human gut microbiome, Nature 505 (2014) 559–563.
[10] W. Fan, Y. Tang, Y. Qu, F. Cao, G. Huo, Infant formula supplemented with low protein and high carbohydrate alters the intestinal microbiota in neonatal SD rats, BMC Microbiol. 14 (2014) 279.
[11] C. Shortt, O. Hasselwander, A. Meynert, et al., Systematic review of the effects of the intestinal microbiota on selected nutrients and non-nutrients, Eur. J. Nutr. 57 (2018) 25–49.
[12] A.J. Richardson, N. McKin, R.J. Wallace, Ammonia production by human faecal bacteria, and the enumeration, isolation and characterization of bacteria capable of growth on peptides and amino acids, BMC Microbiol. 13 (2013) 6.
[13] A.J. Vince, S.M. Burridge, Ammonia production by intestinal bacteria: the effects of lactose, lactulose and glucose, J. Med. Microbiol. 13 (1980) 177–191.
[14] A. Ramezani, Z.A. Manly, B. Meijers, P. Evenepoel, R. Vanholder, D.S. Raj, Role of the gut microbiome in uremia: a potential therapeutic target, Am. J. Kidney Dis. 67 (2016) 486–496.
[15] W. Al-Zyoud, A. Naesseredin, H. Aljarranah, M. Saket, Culturable gut bacteria lack Echerichia coli in children with phenylketonuria, New Microbes New Infect 32 (2019), 100616.
[16] F. Pinede de Oliveira, R.H. Mendes, P.T. Dobbler, et al., Phenylketonuria and gut microbiota: a controlled study based on next-generation sequencing, PLoS One 11 (2016), e0157513.
[17] R.D. Hills Jr., B.A. Pontefract, H.R. Mishcon, C.A. Black, S.C. Sutton, C.R. Thetheberge, Gut microbiome: profound implications for diet and disease, Nutrients 11 (2019).
[18] T.C. Shen, L. Alenberg, K. Bittinger, et al., Engineering the gut microbiota to treat hyperammonemia, J. Clin. Invest. 125 (2015) 2841–2850.
[19] R. Rivera-Flores, S. Moran-Villota, L. Cervantes-Barragan, C. Lopez-Macias, M. Uribe, Manipulation of microbiota with probiotics as an alternative for treatment of hepatic encephalopathy, Nutrition 73 (2020), 110693.
[20] J.T.A. Haberle, E. Sawicki, M. Mahowald, B. Meehan, A. Becarelli, K. Weber, M. J. Koziel, An open-label, single-arm clinical study to evaluate safety and tolerability of SB195, a novel glycine in patients with urea cycle disorders, J. Inherit. Metab. Dis. 42 (2019) 242.
[21] C. Depommier, A. Eversard, C. Druart, et al., Supplementation with akkarmanin mucinophilia in overweight and obese human volunteers: a proof-of-concept exploratory study, Nutr. Med. 25 (2019) 1596–1103.
[22] G. Allegri, S. Deplazes, N. Riman, et al., Comprehensive characterization of ureagenesis in the spllash mouse, a model of human ornithine transcarbamylase deficiency, reveals age-dependency of ammonia detoxification, J. Inherit. Metab. Dis. 42 (2019) 1064–1076.
[23] G. Bassanini, C. Ceccarini, F. Borgo, et al., Phenylketonuria diet promotes shifts in microbial enterotypes, Science 334 (2011) 105–1074.
[24] R. Farre, M. Fiorani, S. Abdu Rahiman, G. Matteoli, Intestinal permeability, inflammation and the role of nutrients, Nutrients 12 (2020).
[25] S. De Baere, V. Eercracht, M. Steppe, et al., Development of a HPLC-UV method for the quantitative determination of four short-chain fatty acids and lactic acid produced by intestinal bacteria during in vitro fermentation, J. Pharm. Biomed. Anal. 80 (2013) 107–115.
[26] eetmeter, Accessed 10-04-2021, 2021, at, https://mijn.voedingscentrum.nl/nl/eetmeter.
[27] P.J. van Spronsen, A.M. van Wegberg, K. Abirn, et al., Key European guidelines for the diagnosis and management of patients with phenylketonuria, Lancet Diabetes Endocrinol. 5 (2017) 743–756.
[28] V.J. Mancilla, A.E. Mann, Y. Zhang, M.S. Allen, The adult phenylketonuria (PKU) gut microbiota, Microorganisms 9 (2021).
[29] L.V. Hooper, D.R. Littman, A.J. Macpherson, Interactions between the microbiota and the immune system, Science 336 (2012) 1268–1273.
[30] A. Gotfliard, S.P. Kennedy, L.C. Kong, et al., Dietary intervention impact on gut microbial gene richness, Nature 500 (2013) 585–588.
[31] H.J. Flint, K.P. Scott, P. Louis, S.H. Duncan, The role of the gut microbiota in nutrition and health, Nat Rev Gastroenterol Hepatol 9 (2012) 577–589.
[32] Y. Zhao, G. Tian, D. Chen, et al., Dietary protein levels and amino acid supplementation patterns alter the composition and functions of colonic microbiota in pigs, Animal Nutrition 6 (2020) 143–151.
[33] D. Radjabzadeh, C.G. Boer, S.A. Beth, et al., Diversity, compositional and functional differences between gut microbiota of children and adults, Sci. Rep. 10 (2020) 1040.
[34] A. Amaretti, C. Gossoli, M. Simone, et al., Profiling of protein degraders in cultures of human gut microbiota, Front. Microbiol. 10 (2019) 2614.
[35] E. Pesini, Lactic acid bacteria contribution to gut microbiota complexity: lights and shadows, Front. Cell. Infect. Microbiol. 2:86– (2012).