ORIGINAL ARTICLE

Culturable gut bacteria lack *Escherichia coli* in children with phenylketonuria

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Abstract

Phenylketonuria (PKU) is an inherited metabolic disorder that affects phenylalanine metabolism. If left untreated, phenylalanine builds up to harmful levels in the body and may cause intellectual disability and other serious health problems. The aim of this study was to compare the culturable predominant bacteria in the gut of PKU versus non-PKU children in Jordan to measure the effect of a PKU low-protein diet on the normal flora. *Escherichia coli* is a bacterium of the normal gut flora in humans and vitally benefits the hosts in producing vitamin B_2 (riboflavin) and vitamin K_2 (menaquinone) involved in human cellular and bone metabolism, respectively. For a small-scale observational study, stool samples were collected from 25 children divided into 20 subjects without PKU as controls and five PKU subjects. Only predominant culturable bacteria were isolated from the stool on CLED (cysteine–lactose–electrolyte-deficient) agar, which was a limitation of this study. Samples were incubated at 35 ± 2°C, observed after 24–48 h, and transported to an automated microbial analyser. Data analysis was obtained using the independent sample t-test to determine any statistically significant difference in the microbial gut community between the associated population means. It was statistically significant ($p < 0.01$) that *E. coli* was present in all control subjects, while it was absent from the gut flora of all PKU subjects. Additional studies on a larger scale are needed to confirm these results and also any association with blood serum levels of phenylalanine and vitamins B_2 and K_2.

Keywords: Children, *E. coli*, Gut, Microbial diversity, Phenylketonuria
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Introduction

Phenylketonuria (PKU) is a rare genetic autosomal recessive disease that results in an error in phenylalanine metabolism. Neurotoxic phenylalanine accumulation can be the root cause of several disorders, such as mental retardation, speech disturbance, seizures, musty skin smell, and poor skin pigmentation. The typical conversion of phenylalanine to tyrosine, a vital component in neurotransmitters, occurs by the enzyme phenylalanine hydroxylase (PAH), which catalyses the hydroxylation of the phenyl group of phenylalanine to transform it to tyrosine. This hydroxylation system consisting of the PAH enzyme itself, the cofactor tetrahydrobiopterin (BH4), an oxygen molecule, and NADPH oxidase [1].

Infants with PKU are usually healthy at birth since the mother's body breaks down phenylalanine during pregnancy. A protein-deficient diet should be implemented as soon as the patient is diagnosed and will be lifelong [2].

The functional genes and metabolites of the human microbiome affect human physiology and are vital factors for eluding diseases. In the past decade, much has been discovered regarding the diversity, structure, stability, and dynamics of the human microbiota within the gastrointestinal tract [3–9]. Variations in lifestyle during different stages of life and in different geographical locations have a significant effect on the gastrointestinal tract microbiome; consequently, these studies may prove helpful in identifying generalizable trends over a human lifetime. Hence, the human gut microbiome is considered as the next big frontier in medicine.

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In recent years, research has shown that gut bacteria influence the immune and endocrine systems, brain health, mood and cognitive function, and several other biological processes [7]. An equilibrium state of good and bad bacteria in the gut maintains health, and imbalance can contribute to disease. Learning how to influence the microbiome to treat disease would contribute to the next medical frontier [5]. For example, vitamin B_2 (riboflavin) undertakes a fundamental role in cell metabolism, acting as a precursor for the coenzymes flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), the hydrogen transporters in numerous organic redox reactions [10]. Riboflavin is biosynthesized in *Bacillus subtilis* and *Escherichia coli* (a facultatively anaerobic, Gram-negative, rod-shaped bacterium of the normal gut flora in humans [11]) from the precursors GTP and D-ribulose 5-phosphate in seven enzymatic steps [12]. Furthermore, vitamin K_2 (menaquinone) can be produced only by gut bacteria, of which *E. coli* is a good example [13]. An adequate dietary intake of vitamin K_2 has been associated with the prevention of coronary heart disease (CHD) [14].

When healthy, the gut microbiota and the human host share a mutualistic relationship in which each benefits from the other. The intestine of the host provides the bacteria with an environment in which to grow, and the bacterial ecosystem maintains homeostasis within the host by modulating physiological functions. Any change in bacterial composition is called dysbiosis or dysbacteriosis, which may lead to an impaired microbiota and can cause a spectrum of diseases (e.g. colorectal cancer) [15]. Variations in the intestinal microbiota have been correlated with the pathophysiology of irritable bowel syndrome [8]. Some of the observed alterations include a decrease in *Lactobacilli* and *Bifidobacteria* and an increase in mucosal bacteria. Moayyedi et al., in a systematic review, have examined 1650 patients suffering from irritable bowel syndrome to whom probiotic treatment was administered; however, the most active species and strains or even the magnitude of the benefit were uncertain in that review [16]. Recently, it was seen that some probiotic administration improved the overall symptoms of irritable bowel syndrome. Various types of probiotics were used –*Lactobacillus, Bifidobacterium, Streptococcus*, and a combination of probiotics –and all showed a trend towards benefit [8].

In 2011, MacDonald and others investigated the influence of adding prebiotic oligosaccharides to a metabolic formula for infants with PKU, and found that the metabolic formula was well tolerated. Advantages of these investigations are the maintenance of metabolic control and *Bifidobacterium* levels and lowered stool pH levels. No significant change was observed in *Bifidobacterium* or lactobacilli–enterococci from baseline levels, but there was a noticeable increase in *Bifidobacterium* levels in two subjects who had the lowest concentrations of *Bifidobacterium* at baseline (3.6% and 6.7% at baseline, increased by 54.6% and 27.9%) [17].

Hence, the study of gut bacterial diversity can be critical in determining which bacteria are missing in PKU subjects, and whether a faecal transplant is a possible treatment plan. The aim of this pilot study was to characterize the changes in the culturable gut microbial community in five subjects with PKU and compare them with 20 subjects without PKU.

Materials and methods

The methodology of the study included first, a questionnaire designed to acquire demographics and other lifestyle and technical data from 20 controls and five PKU subjects as summarized in Table 1. The administration of the questionnaire of diverse sections was designed alongside subject consent forms. The questionnaire sections were adapted from a previously reported study and included weight, height, gender, gender ratio, colour, companion animals, related parents, mode of birth, prebiotics, probiotics, antibiotics, primary vaccination, breast-milk feeding, and solid food [18]. As exclusion criteria, each control and PKU subject had no antibiotic use for at least 3 months before the study to guarantee that any bacterial absence was not due to antibiotics.

Second, a stool sample was collected from each of the 20 controls who were not on a low-protein diet and the five PKU subjects who were on a low-protein diet. Data analysis by the two-tailed t-test was then applied to compare the results from the PKU and control subjects. A significant difference was considered at $p < 0.01$ and a confidence interval of 0.99. The two-tailed t-test was performed by using GraphPad software (Prism V8.2.0, San Diego, USA). It was noticed that the Ho hypothesis is rejected only for *E. coli* but accepted for all other bacterial strains (Table 2).

Bacteria were isolated on CLED agar (*Himedia*, Mumbai, India) and incubated at 35 ± 2°C, observed after 24-48 h, and transported to the Vitek™ 2 compact automated microbial analyser (*bioMérieux*, Lyon, France). The Vitek™ 2 compact system is capable of accurate microbial identification and antibiotic susceptibility testing that uses an extensive identification database for rapid results and minimal training time. Automated technology in microbiology has proved extremely advantageous in recent years.

CLED agar was used for the stool growth culture since several researchers found that its productivity is similar to that of standard procedures (blood agar and MacConkey agar combined), making it a medium worth using for routine culture of stool samples. CLED was used in this study since it is a good growth medium for both pathogenic and non-pathogenic bacteria.

TABLE 1. A questionnaire-based data tabulated for the demographics and historical data of the study subjects

Questionnaire sections	Non-PKU subjects (n = 20)	PKU subjects (n = 5)	P value	Statistically significant at P < 0.01
Mean of weight (kg)	15.19	14.16	0.9149	no
Mean of height (cm)	86.14	73.90	0.5291	no
Mean of age (months)	47.75	61.60	0.7540	no
Gender	11 males	3 males		
	9 females	2 females		
Gender ratio [m/(m + f)]	55%	60%	0.9725	no
Color	11 dark (55%)	0 dark (0%)		
	1 very dark (5%)	0 very dark (0%)		
	5 medium (25%)	0 medium (0%)		
	3 light (15%)	0 light (0%)		
	0 very light (0%)	5 very light		
Companion animal	8 yes (40%)	0 yes (0%)		
	12 no (60%)	5 no (100%)		
Related parents	6 yes (30%)	4 yes (80%)		
	14 no (70%)	1 no (20%)		
Mode of birth	2 natural at home (10%)	0 natural at home (0%)		
	11 natural at hospital (55%)	3 natural at hospital (60%)		
	6 natural at hospital (with pain management) (30%)	2 natural at hospital (with pain management) (40%)		
	1 Cesarean section (5%)	0 Cesarean section (0%)		
Dietary prebiotics	13 yes (65%)	5 yes (100%)		
	7 no (35%)	0 no (0%)		
Probiotics	8 yes (40%)	0 yes (0%)		
	12 no (60%)	5 no (100%)		
Weekly Antibiotics	0 yes (0%)	0 yes (0%)		
<3 months of the study	20 no (100%)	5 no (100%)		
Basic vaccinations	20 yes (100%)	5 yes (100%)		
	0 no (0%)	0 no (0%)		
Breast milk feeding	7 yes (35%)	3 yes (60%)		
	13 no (65%)	2 no (40%)		
Solid food	8 yes (40%)	2 yes (40%)		
	12 no (60%)	3 no (60%)		
Average colony number	15	15		
Average number of bacterial species	12	12		
Vitamins rate/deficiencies	n/a	n/a		
E.coli negative	0 out of 23 (0%)	5 out of 5 (100%)		

This work involved the use of human subjects and was carried out according to the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Informed consent with privacy rights was obtained for experimentation.

Results

No statistical significance was revealed from the two study groups for mean age, gender ratio, mean weight, and mean height. The following observations were recorded: (a) the skin pigmentation of all PKU subjects was poor, very light, which is because the improper metabolism of phenylalanine will lead to the lack of its products involved in the production of melanin [19]; (b) 40% of control subjects had companion animals, whereas none of the PKU subjects did, which might affect microbial diversity [20]; (c) 80% of PKU subjects had related parents, which is consistent with PKU being a genetically inherited disease; (d) the mode of birth, probiotics, antibiotics, primary vaccination, breast-milk feeding, and solid food were inconclusive except that 100% of PKU subjects were on dietary

TABLE 2. The data of the percentages (means) of the presence of only culturable and predominant bacteria obtained from the *Vitek* system as a comparison between the PKU and non-PKU subjects

Phylum	Class	Species	Non-PKU subjects (20)	PKU subjects (5)	P value	Statistically significant at P < 0.01
Proteobacteria	*Gammaproteobacteria*	*E. coli*	96.15%	0.00%	<0.0001	yes
		P. fluorescens	4.70%	0.00%	0.6274	no
		S. fonticola	4.95%	18.80%	0.3101	no
		A. salmonicida	0.00%	17.20%	0.0428	no
		P. stutzeri	4.55%	18.80%	0.2749	no
	Alphaproteobacteria	*S. paucimobilis*	4.75%	0.00%	0.6274	no
		Methylobacterium	0.00%	18.80%	0.0428	no
		R. radiobacter	0.00%	19.20%	0.0428	no
Firmicutes	*Bacilli*	*Lactobacillus*	9.00%	0.00%	0.3769	no
		E. gallinarum	14.20%	0.00%	0.3770	no
	Clostridia	*C. difficile*	4.45%	0.00%	0.6274	no

prebiotics; (e) the average bacterial colony number was 15 for all study subjects, the average number of bacterial species was 12 for all study subjects, and there were no data on the serum vitamins rate/deficiencies in this study (it has been reported in the literature that vitamins B_2 and K_2 are less physiologically available in PKU patients [21,22]); (f) a significant statistical difference was observed only for *E. coli*. All PKU patients were negative for *E.coli*, and all of the controls were *E.coli*-positive. Using the Vitek system, the bacteria identified from the stool samples of controls and PKU subjects are presented in Table 2 and included *E. coli*, *Pseudomonas fluorescens*, *Serratia fonticola*, *Aeromonas salmonicida*, *Pseudomonas stutzeri*, *Sphingomonas paucimobilis*, *Methylobacterium*, *Rhizobium radiobacter*, *Lactobacillus*, *Enterococcus gallinarum* and *Clostridium difficile*. Table 2 shows the bacteria present as percentages of culturable predominant bacteria obtained from the Vitek system, and it correctly identified *E.coli* in 88–99% of each control subject; this enabled us to compare the two populations of PKU and control subjects. However, the stool of PKU patients and non-PKU controls showed similar diversity on the CLED agar plates. From Table 2, it can be seen that PKU subjects also lack the members of the Firmicutes (*Lactobacillus*, *Enterococcus gallinarum* and *Clostridium difficile*) found in the non-PKU subjects.

Discussion

A limitation of this study is that only five subjects with PKU were collected due to the low frequency of PKU and the lack of a recent update on PKU prevalence in Jordan. The means of two independent groups were calculated to determine the statistical evidence on whether the associated population means are significantly different. In this small-scale observational study, it was statistically significant that *E. coli* was present in all control subjects but absent from the gut flora in all PKU subjects. The absence of *E. coli* may have some effect on the ability of the PKU patients to generate vitamin B_2 (riboflavin) [10] and vitamin K_2 (menaquinone) [13] which are normally produced by *E. coli* and are involved in many physiological roles in the human cells, as was found in 96.15% of non-PKU subjects. Vitamin B_2 helps the cells to break down carbohydrates and proteins to produce energy; it is also involved in hydrogen transport [23]. On the other hand, vitamin K_2 is involved in blood clotting and bone growth; it also helps in lowering the risk of developing coronary heart disease [24].

The significant absence of *E. coli* in the PKU population highlights a possible alarming absence of both vitamin B_2 and K_2. From the results in Table 2, PKU subjects also showed a lack of any members of the Firmicutes found in the non-PKU controls. The shift in Firmicutes in this study is consistent with results reported in 2019 by Bassanini et al., based on the sequencing of 16S rRNA gene from PKU faecal samples [25]. From the results in Table 1, it is also noted that all PKU subjects were on proper dietary prebiotics that supposedly enhance the growth of some microorganisms in their guts with low protein intake.

No PKU subjects were on any antibiotic treatments for at least the 3 months prior to the study. All of this information is important to increase the reliability of the study findings by excluding any external factors as much as possible.

E.coli normally represents around 0.1% of the gut flora in humans [26]. The most common *E.coli* strains in the human gut are *E. coli* HS, *E. coli* UTI89, *E. coli* CFT073, *E. coli* KO11FL, *E. coli* NA114, *E. coli* 536, *E. coli* O127: H6 str. E2348/69 [27]. Scientists have already researched the microbiota of PKU patients based on culturomics or metagenomics [3,17,28]; however, to our knowledge no clinical research has been reported on *E.coli* in PKU patients, and only limited data are available on its potential effectiveness as a tool to engineer the human gut microbiota for various health conditions [29]. Other challenges are the ability to set up and differentiate the baseline of healthy microbiomes, and the deviations that occur there.

It is worth mentioning that the use of CLED medium was a limitation of this study. CLED has a good discrimination of Gram-negative bacteria due to lactose fermentation and colony appearance. Nevertheless, it poorly discriminates Gram-positive bacteria due to inhibition of the swarming of *Proteus* species; this might be linked to that fact that CLED lacks sodium chloride, which helps in preventing the swarming phenomenon, making it challenging to isolate microorganisms from a mixture of bacterial species [30] such as are usually present in urinary tract infections. CLED does have a relatively low cost compared to other growth media [19]. Although CLED is not the optimum growth medium for Gram-positive bacteria, this study showed that several Gram-positive bacteria could be identified, including *Lactobacillus*, *Clostridium difficile*, and *Enterococcus*.

Conclusions

Generally, the gut microbiome has an essential role in providing amino acids to the host to maintain homeostasis. The nutritional composition of PKU diets might alter gut microbial ecology and disturb the normal physiology. The findings of this study showed that *E. coli* was significantly absent from the gut of PKU subjects. Additional studies on a larger scale are needed to confirm these results. It is also recommended that further studies elaborate on such rare disorders to improve the quality of life of patients, especially as we know that the accumulation of phenylalanine in the cells of PKU patients may drastically lead to mental retardation and sometimes death. This study was a

pilot, and with limited statistical power; however, the preliminary data of the study have generated an interesting insight into blood serum levels of phenylalanine and the levels of vitamins B_2 and K_2 in PKU patients.

Transparency declaration

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Author contributions

Conceptualization: W.A.-Z,; methodology: W.A.-Z.; formal analysis: A.N. and H.A.; investigation: A.N.; resources: W.A.-Z.; data curation, H.A.; writing (original draft preparation): W.A.-Z., M.S., A.N. & H.A.; writing (review and editing): W.A.-Z. and M.S.; visualization: A.N. and H.A.; supervision: W.A.-Z. and M.S.; project administration: W.A.-Z.; funding acquisition: W.A.-Z.

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