East African Medical Journal Vol. 99 No. 11 November 2022
EFFECTS OF *SOLANUM NIGRUM* ON CAECAL MICROBIOME OF HIGH FAT FED RATS IN A RANDOMIZED CONTROL STUDY
Kathryn Wanjiku Nderitu, Department of Biochemistry, Faculty of Science and Technology, University of Nairobi, P.O Box 30197 -00100, Nairobi, Kenya, Ezekiel Mecha, Department of Biochemistry, Faculty of Science and Technology, University of Nairobi, P.O Box 30197 -00100, Nairobi, Kenya, Stephen Obol Opiyo, The Ohio State University, Columbus, Ohio 4301, United States of America, Atunga Nyachieo, Institute of Primate Research, Karen-Nairobi, Kenya

Corresponding author: Kathryn Wanjiku Nderitu, Department of Biochemistry,University of Nairobi, P.O Box 30197 -00100,Nairobi, Kenya. Email: knderitu276@ gmail.com

EFFECTS OF *SOLANUM NIGRUM* ON CAECAL MICROBIOME OF HIGH FAT FED RATS IN A RANDOMIZED CONTROL STUDY

K. W. Nderitu, E. Mecha, S. O. Opiyo and A. Nyachieo

ABSTRACT

***Background*: Diet influences gut microbiota which in turn affects both metabolism and overall human health. *Solanum nigrum* is an African leafy vegetable which has previously been shown to have both nutritional and medicinal value. However, its effect on gut microbiome has not been elucidated.**
***Objective*: To evaluate the effect of *Solanum nigrum* on gut microbiome.**
***Methods*: *Solanum nigrum* leaves were collected from Kiambu County Kenya, which were later tested on Sprague Dawley high fat fed rats randomized and divided into 7 groups of n=5 rats for 11 weeks to determine its effect on abundance and diversity of the gut microbial community.**
***Results*: All groups had Campylobacterota, Firmicutes, Proteobacteria, Actinobacterota, Bacteriodota, Deferribacterota, Spirochaetota but with varying amounts.**
***Conclusion*: *Solanum nigrum* extract at different dosages had similar effect on the microbiome as that of the standard obesity drug (Orlistat) and could be used as an anti-obesity treatment.**

INTRODUCTION

The diversified community of microorganisms which include bacteria, viruses, archaea, and eukaryotic microbes are known as human microbiota since they co-inhabit on human body surfaces[1]. However, all individuals have unique set of microorganisms on different body parts[2]. There are various reasons for microbial diversity which include: genetic background, lifestyle, geographical location, age, diet, exposure to antibiotics or prebiotics and early exposure to various microorganisms for example during the gestation period, delivery, hospitalization, and during feeding[3]. The gut microbiome plays a key role in human health and disease. They assist in food processing, protection from pathogens, vitamin synthesis, shaping the immune and nervous system, gut epithelium development, and metabolism. The imbalance of the gut microbiome is known as dysbiosis and may lead to host dysfunction thus contributing to the pathogenesis of a disease[4].The African leafy vegetables such as *Solanum nigrum* have previously been shown to have both nutritional and medicinal value, as well as anti-obesity effects compared to other vegetables due to the phytochemical compounds present in them; however, their effect on gut microbiome has not been evaluated. Thus, the aim of this study was to study the effects of *Solanum nigrum* on the caecal microbiome of high fat fed Sprague Dawley rats

METHODOLOGY

Study design

This experiment was a randomized controlled study using high-fat fed diet Sprague Dawley rats to evaluate anti-obesity effects and caecal microbiota changes due to administration of *Solanum nigrum* compared with standard drug Orlistat and with normal diet fed Sprague Dawley rats.

Collection of Solanum nigrum

Fresh vegetable leaves of *Solanum nigrum* weighing 2000grams were collected from Limuru sub-County, Kiambu County. They were packed in khaki bags, transported to the department of biological sciences, University of Nairobi for identification and authentication by a taxonomist, and allocated voucher specimen number (KWNUON2019/001). The samples were then transported to the department of chemistry, University of Nairobi for extraction of bioactive compounds using two solvents namely: methanol and dichloromethane.

Extraction of phytochemical compounds

Extraction of bioactive compounds was done at the Chemistry Laboratories, University of Nairobi. This was done by first grinding the leaves into powder using an electric mill. The sample material was soaked twice in 100% dichloromethane (DCM) for 24hours respectively and then re-soaked in 100% methanol for another 48hours. The solvent covered the grounded powder. Whattman number 1 paper was used to filter the mixture and the filtrate obtained was concentrated by rotary evaporator at 39^{o}C and 64^{o}C respectively. The concentrate was used for bioassay [5-6]

Laboratory Animals

Thirty-five (n=35) Sprague Dawley rats weighing 160-180 g were purchased from Kabete veterinary laboratory and transported to the University of Nairobi, Biochemistry Department Animal House. The rats were left to acclimatize for 1 week in standard cages under normal laboratory conditions (25± 2^{o}C, 12 hours light, and 12hours dark cycle) before commencing the experiment. The research protocol was approved by the Institutional Review Committee (IRC) of Institute of

Primate Research on use and care of experimental animal (ISERC/06/19).

High fat diet preparation

The high fat diet was prepared by heating 30g of fat in 100g of rat chow pellet for 20 minutes and monosodium sulphate was added to the feed to add its palatability (Unga Feeds Kenya LTD) [7].

Biological Assay

The male Sprague Dawley rats (n=35) were randomly divided into seven groups with n=5 rats per group where group 1 was given normal diet and groups 2-7 were given high fat diet. The treatment was administered as follows: Group 1 (KWN1) no treatment; Group 2 (KWN2) Orlistat drug of 30mg/kgbw weight; Group 3 (KWN3) no treatment; while group 4 to 7 were given *Solanum nigrum* extracts as follows; Group 4 (KWN4) 150mg/kgbw of MeoH extract; Group5 (KWN5) 300mg/kgbw of MeoH extract; Group 6 (KWN6) 150mg/kgbw of DCM extract and Group 7 (KWN7) 300mg/kgbw of DCM extract respectively (Figure 1).

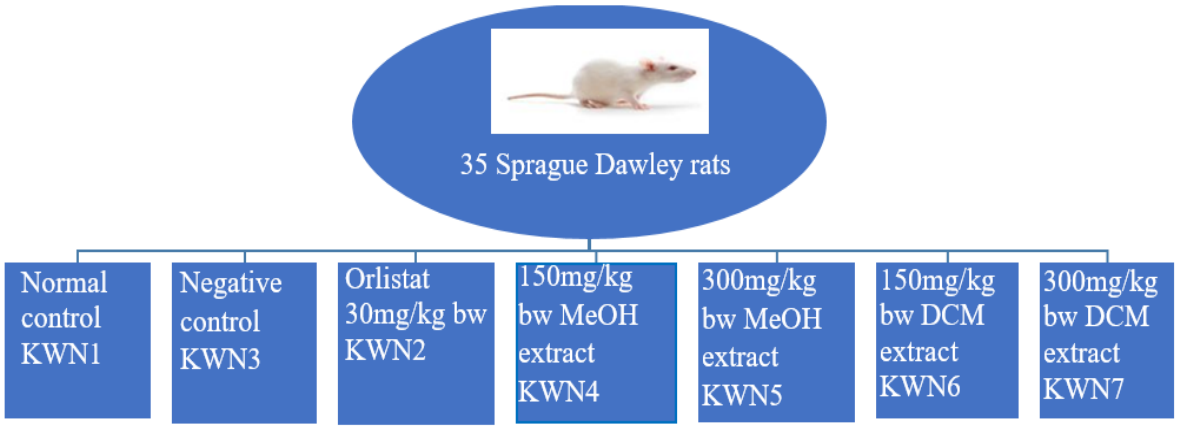

Figure 1: Seven experimental groups with a sample of 5 rats per group. MeoH: Methanol, DCM: Dichloromethane

Microbiota extraction and Sequencing

Rats were sacrificed after eleven weeks of treatment and caecum samples collected at random from each experimental group. The collected samples were subjected to DNA extraction using the ZR fecal DNA Mini Prep per manufacturer's protocol (Zymo Research, California, USA). Caecal samples were lysed by bead beating and DNA isolated using fast spin columns thereafter the DNA pellet was filtered to remove polyphenols and humic acids. DNA quality was determined spectrophotometrically using a NanoDrop™ 2000/2000c spectrophotometer at 260nm/280nm and OD 260nm/230nm. Further DNA quality was confirmed through agarose gel electrophoresis. The DNA pellets were later dissolved in 20 µl TE buffer awaiting PCR assay.

16sr RNA sequencing

The eluted DNA was then shipped to Humanizing Genomics Macrogen in South Korea. Amplification of v3- v4 region was done using 16s rRNA of the normalized DNA using primer515F/806R which targets bacteria and archaea. High through-put-

sequencing was performed with Illumina Miseq paired- end 250 base pairs runs. Amplification of the V3-V4 region of 16s ribosomal RNA gene was done via PCR with the following primers; F515 (5'GTGCCAGCMGCCGCGGTAA -3') and R806 (5' -GGACTACHVGGGTWTCTAAT-3'). The targeted sequences were then demultiplexed and later clustered into operational taxonomic units (OTUs) before taxonomy assignment; thereafter they were analyzed using the bioinformatics pipeline. The analysis was done with the statistical software R version 4.1.3 and R-Studio, analysed sequences were used to determine relative phylum abundance, phylogenetic tree, the alpha and beta diversity. Chi-square test was done to determine statistical significance among the various phylum.

RESULTS

The study showed that change in diet from normal diet to high fat diet lead to obesity and changed the microbiome population of caecum in rats. In addition, administration of *Solanum nigrum* extract at different doses had similar anti-obesity effect and effected similar microbiome changes as that of the standard obesity drug (Orlistat). These findings were supported by the variability of bacterial phyla present in the gut of the various treatment groups as described herein. The various bacteria evaluated included: Actinobacteria, Bacteroidota, Campylobacterota, Deferribacterota, Firmicutes, Proteobacteria and Sprirochaetota. The most prevalent phylum among the groups was Campylobacterota which was above 80% among the following groups KWN2, KWN4, KWN6 and KWN7, followed by Firmicutes and lastly Bacteroidota. The normal control group (KWN1) had the highest percentage of firmicutes (90%) compared to KWN2 (3%), KWN3(40%), KWN4 (1%), KWN5 (25%), KWN6 (5%) and KWN7 (1%). However, the negative control (KWN3) had Bacteroidota (14%) which is insignificantly different from treatment group 5 (KWN5) (18%). One percent to5% of proteobacteria was also present in all the experimental groups. To determine statistical significance among the phyla, Chi-square test was done whose results showed that the phylum was statistically significantly different among the treatment groups (Table1, $p < 0.0001$). *Solanum nigrum* treatment groups had significantly higher composition of Campylobacterota (KWN4-99%; KWN5-55%; KWN6-92%; KWN7-90%) as compared to the negative control (no treatment KWN3-35%) ($p<0.001$) and normal control (KWN5-5%) ($p<0.001$) but similar to positive control (Orlistat treatment KWN2-96%) ($p>0.05$). However, as compared to the normal control (KWN1; no fat diet and no treatment), high fat diet with supplementation of *Solanum nigrum* extracts decreased the intestinal Firmicutes (KWN4-7). Other bacteria (Firmicutes, Bacteriodata, Proteobacteria and Spirochaeta) were comparable among the treatment groups (≤5%) except KWN5 which had higher Firmicutes (25%) and Bacteriodata (18%) ($p<0.001$) (Figure 2). The alpha diversity Shannon and Simpson) indicated significant variations within the treatment groups but no significant difference between KWN3 and KWN5 (Figure 3). Beta diversity showed significant difference between the treatments administered to various groups (Figure 4). Additionally, the results showed that treatment in group 2 (KWN2) was similar to treatment administered to group 7 (KWN7) (Figure 4). On the other hand, there was a

significant difference between treatments administered to group KWN5 and KWN7 (Figure 4). Finally, the cluster dendogram showed various clusters (Figure 5). In general, there were three clusters which showed that KWN1 treatment was not similar to any other treatment groups; treatment in group 2 (KWN2) was similar to treatment group 7 (KWN7) and treatment group KWN5 was similar to treatment group KWN7 (Figure 5).

Table 1

Chi-square test for the goodness of fit for the percentage of the abundance of phylum

Library	Ch-square Test	p-value
KWN1	366.88	< 0.001
KWN2	622.88	< 0.001
KWN3	156.2	< 0.001
KWN4	668.44	< 0.001
KWN5	201.4	< 0.001
KWN6	516.08	< 0.001
KWN7	550.88	< 0.001

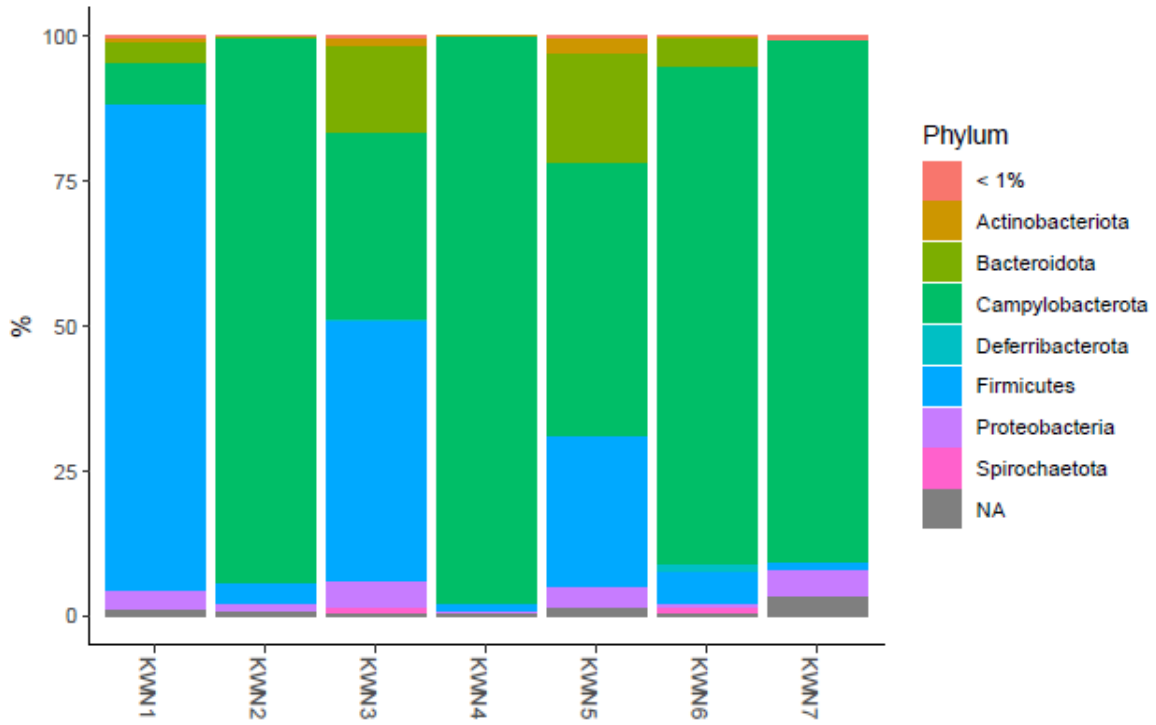

Figure 2: Relative abundance of the microbial population at the phyla level, the different colors indicate the different phyla present

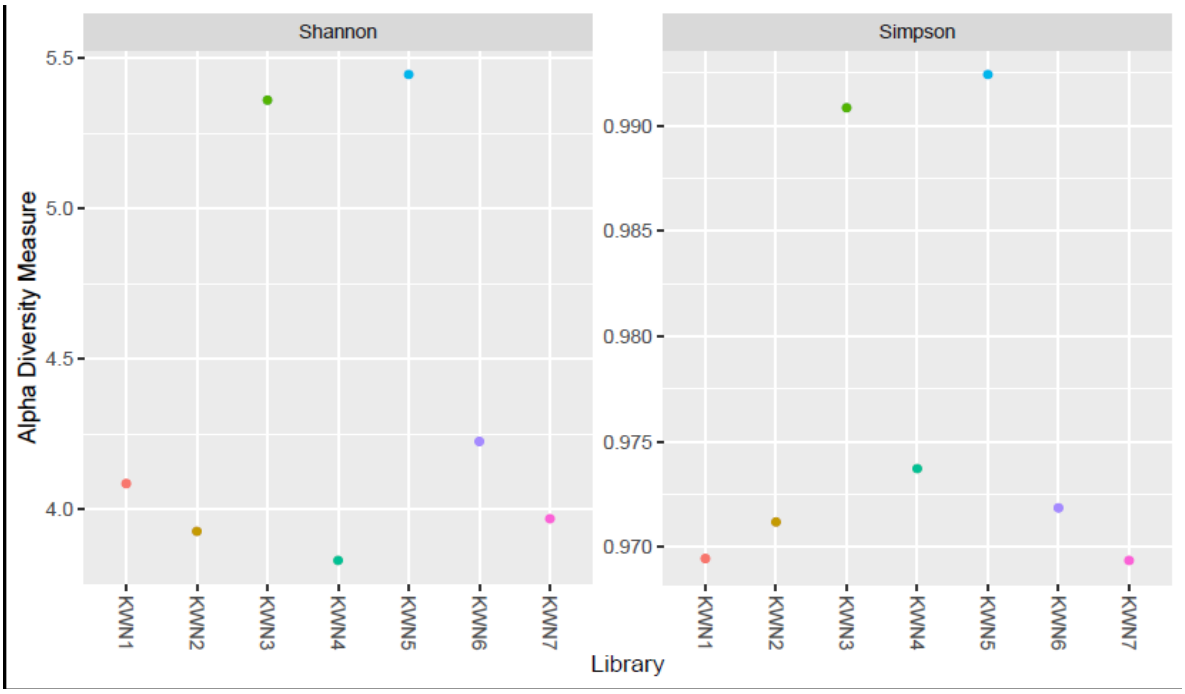

Figure 3: Alpha diversity (Shannon and Simpson) of the different treatment groups

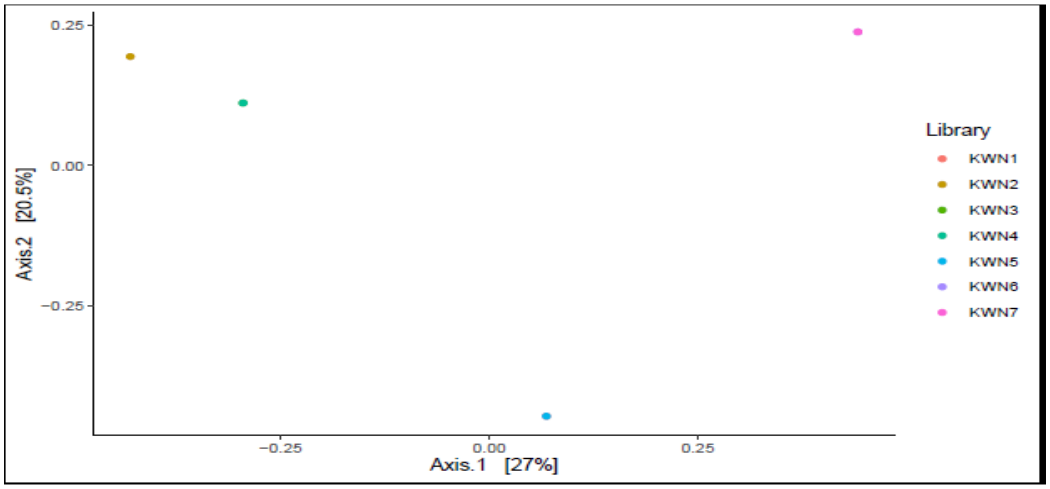

Figure 4: Beta diversity of the different treatment groups

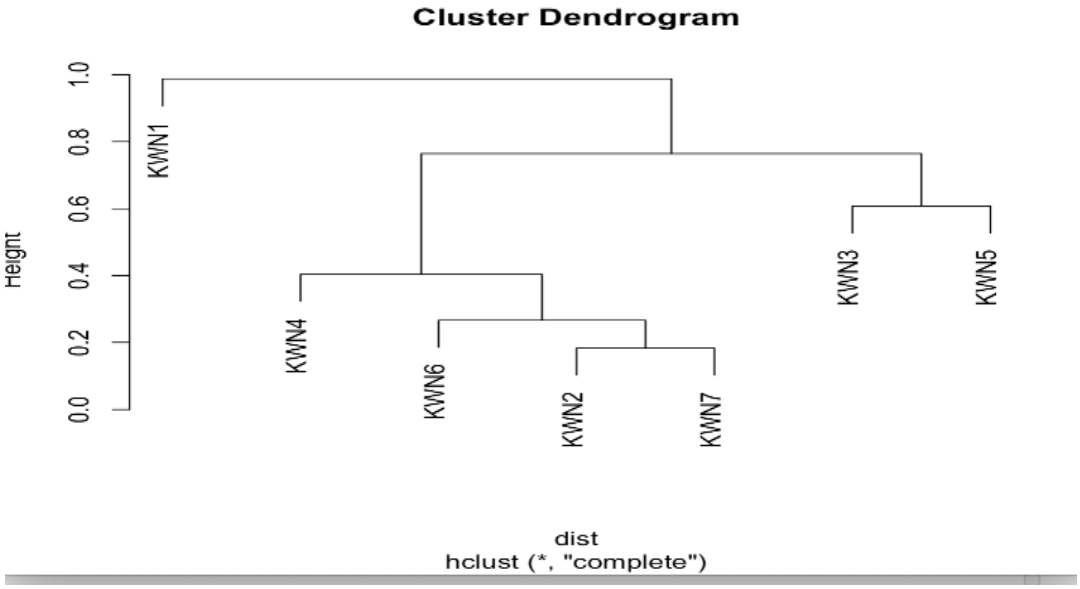

Figure 5: Phylogenetic tree of phyla abundance of the treatment groups

DISCUSSION

The Presence of microbiota may indicate health or disease and may vary according to diet[8–10]. In our study, all groups had Campylobacterota, Firmicutes, Proteobacteria, Actinobacterota, Bacteroidota, Deferribacterota, Spirochaetota but with varying amounts. The presence of these bacteria has been supported by previous studies indicating that Firmicutes, Actinobacteria, Bacteroidota and Proteobacteria are found at the small intestines as well as the colon and caecum [11]. The varying amounts observed may be due to differences in diets provided for each study group. Our study hypothesizes generally, that the presence of these bacteria may have played a role in digestion, synthesis and absorption of nutrients, metabolism of lipids, amino acids, vitamins and short chain fatty acids as supported by previous studies[8,12,13].

Relative proportions varied according to our study groups. For instance, our study showed increased abundance of Campylobacterota on both positive control (Orlistat treated) and *Solanum nigrum* treatment groups. In contrast, this study showed low abundance of Campylobacterota on the negative control and was similar to previous study indicating that consumption of westernized diet composed of high fat diet depletes Campylobacter[14]. We hypothesize that *Solanum nigrum* extracts and Orlistat negatively impact on high fat diets and also provide phytochemicals that help in proliferation of Campylobacterota. Indeed, previous studies indicate that Campylobacterota degrades nitrites in high fat fed dieted animals[15–17]. Nitric oxide plays an important role in endothelial functioning in cardiovascular homeostasis. Its pathway is regulated by nitric oxide synthase such as inducible nitric oxide synthase, endothelial nitric oxide synthase and neuronal nitric oxide synthase. The expression of endothelial nitric oxide synthase increases after long exposure to high fat diet and is an indicator of obesity. On the other hand, previous research showed that hepatic amino acid signaling regulates lipid metabolism through

the neuronal pathway by decreasing adipose lipoprotein lipase expression thus suppressing triglyceride hydrolysis activity. Campylobacterota is known to degrade aromatic amino acids and thus we therefore postulate that its abundance on the treatment groups led to inhibition of the lipase enzyme similar to the Orlistat group[15–17].

Our study further showed high fat diet with supplementation of *Solanum nigrum* extracts decreased the intestinal Firmicutes when compared to the normal control (no high fat diet and no treatment) and negative control group (high fat diet but no treatment) respectively. This is similar to previous study which indicated that introduction of plant polyphenols inhibits growth of firmicutes and bacteroidata by down-regulating firmicutes to bacteroidata ratio [18,19]. Other studies have suggested that firmicutes are known to produce butyrate whichincreases insulin sensitivity and is also known as an energy metabolism regulator. In contrast, although treatment KWN5 was given *Solanum nigrum* methanolic extract of 300mg/kgbw it showed a higher percentage of firmicutes and bacteroidota similar to negative control and comparable to other treatment groups. We postulate that this dose did not have a similar effect as that of Orlistat and thus pancreatic lipase enzyme was not inhibited. This led to an increase in absorption of fatty acids to the adipocytes and thus led to overweight in rats.

Other bacteria such as Proteobacteria, Actinobacterota, Deferribacterota, Spirochaetota were comparable among the treatment groups (≤5%).This study was inline with a study which indicated prevalence of proteobacteria in the intestines of normal control subjects. Proteobacteria are facultative anaerobes whichmake intestinal niche favor the colonization of obligate anaerobes which are laterreplaced by firmicutes and bacteroidetes[20].

Beta diversity and the phylogenetic tree showed treatment administered was significantly different according to the clusters in our study. Generally, there were three clusters; cluster 1 (normal control KWN1), cluster 2 (KWN3 and KWN5) and cluster 3 (KWN 4-7). Cluster 1 was given a normal diet only and hence did not cluster with any other treatment group. On the other hand, cluster 2 had both the high fat diet group (negative control) and treatment group 5 which had both high fat diet and methanolic dose extract of *Solanum nigrum* at 300mg/kgbw. We postulate that the extract did not have an effect on gut microbiome of the rats hence was similar to that of negative control. The other treatment groups clustered together as Cluster 3 since the effect was almost similar on the gut microbiome. This clustering provides further support that the compounds present on the *Solanum nigrum* extracts and Orlistat drug had an effect on the microbial composition compared to the other clusters.

We therefore conclude that change in diet from normal diet to high fat diet changed the microbiome population of caecum in rats. In addition, administration of *Solanum nigrum* extract at different doses had similar effect on the microbiome as that of the standard obesity drug (Orlistat) and could be used as an anti-obesity treatment.

REFERENCES

1.Aoun A, Darwish F, Hamod N. The influence of the gut microbiome on obesity in adults and the role of probiotics, prebiotics, and synbiotics for weight loss. Prev Nutr Food Sci. 2020;25(2):113.

2.Al Khodor S, Reichert B, Shatat IF. The microbiome and blood pressure: can microbes regulate our blood pressure? Front Pediatr. 2017;5:138.

3.Johnson CL, Versalovic J. The human microbiome and its potential importance to pediatrics. Pediatrics. 2012;129(5):950–60.
4.Kachrimanidou M, Tsintarakis E. Insights into the role of human gut microbiota in Clostridioides difficile infection. Microorganisms. 2020;8(2):200.
5.Arika WM, Kibiti CM, Njagi JM, Ngugi MP. Anti-obesity effects of dichloromethane leaf extract of Gnidia glauca in high fat diet-induced obese rats. Heliyon. 2019 Nov 1;5(11):e02800.
6.Zhi C, Huang J, Wang J, Cao H, Bai Y, Guo J, et al. Connection between gut microbiome and the development of obesity. Eur J Clin Microbiol Infect Dis. 2019;38(11):1987–98.
7.Mutiso SK, Rono DK, Bukachi F. Relationship between anthropometric measures and early electrocardiographic changes in obese rats. BMC Res Notes. 2014;7(1):1–7.
8.Meng Y, Li X, Zhang J, Wang C, Lu F. Effects of different diets on microbiota in the small intestine mucus and weight regulation in rats. Sci Rep. 2019;9(1):1–12.
9.Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, et al. Human gut microbiome viewed across age and geography. nature. 2012;486(7402):222–7.
10.Kachrimanidou M, Tsintarakis E. Insights into the role of human gut microbiota in Clostridioides difficile infection. Microorganisms. 2020;8(2):200.
11.Luo L, Zhang J, Liu M, Qiu S, Yi S, Yu W, et al. Monofloral Triadica Cochinchinensis Honey Polyphenols Improve Alcohol-Induced Liver Disease by Regulating the Gut Microbiota of Mice. Front Immunol. 2021;12:1846.
12.Rinninella E, Raoul P, Cintoni M, Franceschi F, Miggiano GAD, Gasbarrini A, et al. What is the healthy gut microbiota composition? A changing ecosystem across age, environment, diet, and diseases. Microorganisms. 2019;7(1):14.
13.Krajmalnik-Brown R, Ilhan ZE, Kang DW, DiBaise JK. Effects of gut microbes on nutrient absorption and energy regulation. Nutr Clin Pract. 2012;27(2):201–14.
14.Masanta WO, Heimesaat MM, Bereswill S, Tareen AM, Lugert R, Groß U, et al. Modification of Intestinal Microbiota and Its Consequences for Innate Immune Response in the Pathogenesis of Campylobacteriosis. Clin Dev Immunol. 2013 Nov 14;2013:e526860.
15.Jandhyala SM, Talukdar R, Subramanyam C, Vuyyuru H, Sasikala M, Reddy DN. Role of the normal gut microbiota. World J Gastroenterol WJG. 2015;21(29):8787.
16.Akram F, Fuchs D, Daue M, Nijjar G, Ryan A, Benros ME, et al. Association of plasma nitrite levels with obesity and metabolic syndrome in the Old Order Amish. Obes Sci Pract. 2018 Oct;4(5):468.
17.Moran-Ramos S, Ocampo-Medina E, Gutierrez-Aguilar R, Macías-Kauffer L, Villamil-Ramírez H, López-Contreras BE, et al. An amino acid signature associated with obesity predicts 2-year risk of hypertriglyceridemia in school-age children. Sci Rep. 2017;7(1):1–9.
18.Parkar SG, Trower TM, Stevenson DE. Fecal microbial metabolism of polyphenols and its effects on human gut microbiota. Anaerobe. 2013;23:12–9.
19.Xue B, Xie J, Huang J, Chen L, Gao L, Ou S, et al. Plant polyphenols alter a pathway of energy metabolism by inhibiting fecal Bacteroidetes and Firmicutes in vitro. Food Funct. 2016 Mar 16;7(3):1501–7.
20.Shin NR, Whon TW, Bae JW. Proteobacteria: microbial signature of dysbiosis in gut microbiota. Trends Biotechnol. 2015;33(9):496–503.