Impact of Interplay between Obese Gut Microbiota and Diet in Developing Obesity in Synthetic Community Mice

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Abstract: This study aims at investigating the effects of cultured gut microbiota (GM) of obese human coupled high fat diet (HFD) or chow diet (CD) in development of obesity in mice. 20 stool samples were collected from obese patients and isolated bacteria were identified morphologically and biochemically. Identified isolates were mixed in equal proportions to synthesize obese GM. In vivo study was performed using obese GM combined with HFD/CD using mouse model for three months. Albino mice were treated with ampicillin from one week prior to birth until weaning of the pups at seven weeks of age and then inoculated with obese GM. Sixteen mice were divided into four groups: i.e. group 1 (G1) mice fed with CD, group 2 (G2) mice with HFD, group 3 (G3) mice with GM + HFD and group 4 (G4) mice with GM + CD. Mice from groups 3-4 were considered synthetic community (SC) mice due to transfer of synthesize human GM. 16S rRNA sequencing identified five abundant bacteria as Pseudomonas aeruginosa, Staphylococcus sp., Escherichia coli, Morganella morganii, and Klebsiella oxytoca (accession numbers: MZ150742-MZ150746). In vivo study indicated that GM combination with either HFD/CD caused significantly increased body weight in SC mice (BMI; Kg/m2) compared to HFD or CD fed mice groups. One way ANOVA revealed highly significant increase (p ≤ 0.001) in levels of total cholesterol (TC), triglycerides and low density lipoprotein (LDL) in GM coupled diet groups (G3-G4; SC mice) compared to significant increase in HFD group (G2) versus CD group (G1). Our study is first of its kind to report significant effects of using purified strains as obese GM plus diet (HFD/CD) in inducing obesity in SC mice and elevated serum liver parameters as metabolic indicators, hence providing strong evidence about significance of modified GM combination with HFD in developing obesity in SC mice.

Key words: gut microbiota, high fat diet, synthetic community mice, obesity, serum liver parameters

1 Introduction

Obesity is a condition in which excess fat accumulate in body to such an extent that it may cause harmful effects on health1. Genetic and environmental factors play important role in development of obesity. Together with environmental factors, increased fat intake or decreased physical activities, obesity may result from changes in energy balance i.e. energy storage and harvest2. Obesity may be linked to development of several serious ailments including hypertension, diabetes, dyslipidemia and hypertriglyceridemia3. Globally more than 1.9 billion adults were overweight and 650 million were considered obese4,5. Obesity linkage with gut microbiota (GM), gut barrier disruption and inflammation has been a matter of debate recently6,7. Various authors have shown a relation between various metabolic diseases particularly obesity and GM6,7. Due to importance of GM as contributing factor in developing obesity8, scientific interest has increased to understand
its role in obesity and associated metabolic diseases including type 2 diabetes, metabolic endotoxemia etc. Using various integrated pathways, GM exerts its role via interaction with host immune system, diet and genome in addition to several environmental factors.

Most of the symbiotic GM of human consists of fungi, bacteria, viruses and micro eukaryotes. Approximately, $10^{14}$ gut microbes (GM) inhabits human gut and continuous changes in diversity of the GM may increase the person’s exposure to diseases like obesity, diabetes, IBDs, asthma etc. Studies revealed that there is a significant link between obesity and certain bacterial groups like Staphylococcus aureus, Lactobacillus sp., Faecalibacterium prausnitzii and Escherishia coli. Three most prominent gastrointestinal phyla of adult human include Gram negative Bacteroidetes, Gram positive Firmicutes and Actinobacteria. Gut of obese human shows higher populations of Firmicutes and lesser Bacteroidetes while more Bacteroidetes and less Firmicutes were found in lean individuals.

Striking differences in gut microbiome of mouse and human have been proposed previously with relative abundance of different genera in each of two models. Human gut microbiota has Prevotella, Faecalibacterium and Ruminococcus genera with high abundance compared to mouse GM, where Lactobacillus, Alistipes and Turicibacter are more abundant in addition to Clostridium, Bacteroides and Blautia with similar relative abundance in both organisms. Furthermore, compared to humans, mouse GM was mostly identified using culture independent methods. Diet may be considered as one of the main factor involved in diversified GM. High fat feeding produces alterations in serum lipid parameters, total cholesterol (TC), triglycerides (TG), high density lipoprotein (HDL), low density lipoprotein (LDL) and very low density lipoproteins (VLDL) and increase lipopolysaccharide (LPS) transportation in circulation, thus causing development of various metabolic diseases. Gut microbiota may hydrolyze the indigestible polysaccharides and change it to digestible monosaccharides. This activates lipoprotein lipase causing increased blood glucose level hence causing lipogenesis. As a result, fat excessively deposits in adipose tissues by de novo synthesis of triglycerides, resulting in obesity.

There are still lots of pitfalls regarding the understanding of relationship between GM, diet and obesity. In pursuit of this, we used culture dependent and independent techniques to assess the impact of GM coupled modified diet (HFD/CD) in inducing obesity in mice.

2 Materials and Methods

2.1 Sample collection

All subjects gave fully written informed consent about participation in study which was approved by Institutional Bioethics Committee vide letter no. IBC – 180917, GC University, Lahore. We selected 20 obese volunteers from Mayo hospital Lahore and their stool samples were collected in properly labeled 50 ml sterile stool collection vials. Fresh stool inoculum was prepared by mixing a sample in saline (0.85%) and spread dilutions on different media such as macConkey agar, nutrient agar, tryptone soy agar (TSA) and xylose lysine deoxycholate agar (XLD) for isolation of different gut isolates.

2.2 Morphological, biochemical and genetic characterization of bacterial isolates

Gut isolates from obese humans were characterized morphologically and biochemically using Gram staining along with different biochemical tests such as catalase, citrate utilization, urease, H₂S production, methyl red, indole, voges proskauer and denitrification etc. following gerhardt et al. under aerobic and anaerobic conditions. Genomic DNA of abundantly found gut bacteria was isolated using TIANamp bacterial DNA kit and PCR was performed to amplify 16S rRNA gene under standard conditions in a thermal cycler using Universal primers 16S-27-F (5’AGAGTTTGATCCTGGCTCAG-3’) and 16S-1522-R (5’-AAGGAGGTGATCCACGCGCA-3’). The amplified product was sent for sequencing to Axil scientific, Singapore. Sequenced data was obtained and examined using BLAST software.

2.3 In vivo study: Animal housing (weaning)

All animals were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Department of Zoology, GC University, Lahore, Pakistan. The study was approved by the Institutional Bioethics Committee via Approval number IBC-180918 following guidelines designed by Committee for the Purpose of control and supervision of experiments on animals, Pakistan. In order to transfer GM from obese humans to albino mice, modification of protocol by ellekilde et al. was followed. In our view, it is a more simple approach to understand the interplay between GM, diet and obesity. Briefly, albino mice were mated and one week before birth, ampicillin (1 g/L) was added to drinking water of mother. 16 Pups were selected and treated with ampicillin treated water till the age of seven weeks. Afterwards, human GM was used to supplement mice feed to induce obesity.

2.4 Mouse study and diets

Two types of diet were used, standard chow diet (CD) providing energy as 20% protein, 70% carbohydrate and
Table 1  Biochemical characterization of bacteria from obese patients.

| Bacterial strains | Citrate | H₂S Pro. | Urease | Cat. | Nit. Red. | VP | Starch | MR | Indole | Carbohydrate Fermentation | Inference |
|-------------------|---------|----------|--------|------|-----------|----|--------|----|--------|---------------------------|-----------|
|                   |         |          |        |      |           |    |        |     |        | Glucose | Sucrose | Lactose |                   |
| OPGM1             | +       | -        | +      | +    | +         | +  | +      | +  | -      | +        | +        | +       | Staphylococcus sp. |
| OPGM2             | -       | -        | -      | -    | -         | -  | -      | -  | -      | -        | -        | -       | Escherichia sp.   |
| OPGM3             | +       | -        | -      | +    | +         | -  | -      | -  | -      | -        | -        | -       | Pseudomonas sp.   |
| OPGM4             | +       | -        | +      | +    | +         | +  | +      | +  | -      | +        | +        | +       | Staphylococcus sp.|
| OPGM5             | -       | -        | -      | -    | -         | -  | -      | -  | -      | -        | -        | -       | Lactobacillus sp. |
| OPGM6             | -       | -        | -      | +    | -         | +  | -      | -  | -      | -        | -        | -       | Escherichia sp.   |
| OPGM7             | +       | -        | +      | +    | +         | +  | -      | -  | -      | -        | -        | -       | Klebsiella sp.    |
| OPGM8             | -       | -        | +      | +    | -         | -  | +      | +  | +      | +        | +        | -       | Morganella sp.    |
| OPGM9             | +       | -        | -      | +    | +         | -  | -      | -  | -      | +        | +        | +       | Enterobacter sp.  |
| OPGM10            | +       | -        | +      | +    | +         | +  | -      | -  | -      | +        | +        | +       | Klebsiella sp.    |
| OPGM11            | -       | -        | +      | -    | +         | -  | +      | +  | +      | +        | +        | -       | Morganella sp.    |
| OPGM12            | +       | -        | +      | +    | +         | +  | -      | -  | -      | +        | +        | +       | Klebsiella sp.    |
| OPGM13            | +       | -        | +      | +    | +         | +  | -      | -  | -      | +        | +        | +       | Staphylococcus sp.|
| OPGM14            | +       | -        | -      | +    | -         | -  | -      | -  | -      | -        | -        | -       | Pseudomonas sp.   |
| OPGM15            | -       | -        | -      | +    | +         | -  | +      | +  | -      | +        | +        | +       | Lactobacillus sp. |
| OPGM16            | +       | -        | -      | +    | +         | -  | -      | -  | -      | -        | -        | -       | Pseudomonas sp.   |
| OPGM17            | +       | -        | +      | +    | +         | +  | +      | +  | +      | +        | +        | +       | Staphylococcus sp.|

OPGM, Obese patients gut microbes; H₂S Pro., H₂S Production; Cat., Catalase; Nit. Red., Nitrate reduction; VP., Voges proskauer;
10% fat, high fat diet (HFD) providing energy as 13% protein, 6% carbohydrate and 81% fats\textsuperscript{20}. To prepare GM as mice feed inoculum 16S rRNA gene identified gut isolates were grown overnight, adjusted to OD = 1 ± 0.4 in the morning, mixed equally followed by 1:10 dilution in a 50% glycerol solution. These were divided into small aliquots and stored at −80°C. At the day of inoculation, further dilution was made in ratios of 1:5 and 0.15 ml was given to each mouse of experimental groups\textsuperscript{30}. Sixteen 7 week old mice with similar body weights were used for study observation of 12 weeks period as established by Xu et al.\textsuperscript{22} and randomly distributed into following 4 groups (04 mice per group; 2 males and 2 females): Group 1 (G1) fed with chow diet (CD), group 2 (G2) with HFD (n = 4), group 3 (G3) with GM + HFD, group 4 (G4) with GM + CD. The last two groups (G3, G4) were named synthetic community (SC) mice groups. All mice were caged and maintained in sterile cages with autoclaved chip bedding at temperature of 21°C ± 2°C, 55% ± 10% relative humidity and standard 12-hrs per night/day cycle. Body mass index (BMI) was calculated using formula [body weight (g) × 1000/body length (cm)]\textsuperscript{13}.

2.5 Study of serum lipid parameters

After 12 weeks period, all mice were sacrificed by cardiac puncture. Blood was collected in EDTA tubes and allowed to clot for 30 min followed by centrifugation at 3000 rpm for 15-20 minutes. Serum was collected and stored at −80°C until analysis. Serum total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL) of the mice were measured\textsuperscript{23}.

2.6 Microbial diversity of fecal and cecal samples

Fecal pellets were collected at first day of experiment and at the end of week 12 from each of the four mice groups (G1-G4). These were collected on ice and stored at −80°C. Cecal contents were also collected on dry ice aseptically from intestine of two randomly selected sacrificed mice and stored at −80°C. Morphological and biochemical characterization of fecal and cecal bacteria was performed aerobically and anaerobically.

2.7 Statistical analysis

Statistical analysis was done using SPSS statistical software (Version 13.0). All values were expressed as means ± SEM. Paired T-test and one-way ANOVA were applied to analyze data. \( P \) value of ≤ 0.05 was considered statistically significant.

3 Results

3.1 Gut microbiota of obese humans

Initially, from 20 obese patients, a total of 30 visibly different bacteria were isolated. Subsequent purification and biochemical characterization led to 17 isolates only. These strains were found to belong to different genera such as Lactobacillus sp., Kebsiella sp., Escherichia sp., Staphylococcus sp., Pseudomonas sp., Morganella sp. and Enterobacter sp. (Table 1). Further observation of five abundant genera such as Kebsiella sp., Escherichia sp., Staphylococcus sp., Pseudomonas sp., Morganella sp. led us to identify five isolates up to species level. 16S rRNA gene sequencing identified these isolates as Pseudomonas aeruginosa, Staphylococcus sp., Escherichia coli, Morganella morganii and Klebsiella oxytoca (accession numbers: MZ150742-MZ150746). Phylogenetic tree constructed in MEGA X (v10.1.1) using neighbor joining method is shown in Fig. 1.

![Fig. 1](image-url)  Phylogenetic relation of gut microbiota obtained from obese patients. Phylogenetic tree was constructed with MEGA X (v10.1.1) using neighbor joining method.
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3.2 Impact of HFD and GM on BMI of mice

To investigate the role of GM on metabolic parameters of mice, we supplemented these with modified diets (HFD/CD) alone and combined with GM. Significant increase \( (p < 0.001) \) in BMI was observed after 12 weeks of feeding GM plus HFD (G3) and CD (G4) compared to the HFD (G2) and CD fed mice (G1). Furthermore, GM plus HFD/CD fed mice (G3-G4) were also heavier compared to CD and HFD fed mice groups (G1-G2). Overall, BMI as a parameter to indicate obesity in mice was more remarkable in GM plus HFD group (G3) followed by GM plus CD group (G4) and HFD group (G2) compared to CD treated group (G1) (Fig. 2).

3.3 Impact of GM with HFD/CD on serum lipid parameters

We next examined the effect of diets (HFD/CD) alone and combined with GM on various serum biochemical parameters. It was observed that GM plus HFD combination significantly increased TC, triglycerides and LDL, without affecting HDL and VLDL level in group 3 (G3) mice \( (p < 0.001) \). Consistently, the group 4 (G4) mice fed with GM plus CD also showed similar trend. Group 2 (G2) mice showed significant increase in all serum parameters except for the HDL and VLDL where almost similar level was observed compared to CD fed group (G1). Feeding GM plus HFD/CD was likely to aggravate dyslipidemia in mice groups 3-4 mice compared to other CD fed group (G1), though significant \( (p < 0.05) \) increase in HFD fed group was also observed (Fig. 3).

3.4 Modulation of fecal and cecal bacterial diversity

As observed above, highly increased levels of TC, triglycerides and LDL were observed in mice where GM was supplemented with diet, thus we wonder that whether altered serum levels were associated with changes in GM. To determine this, fecal and cecal bacteria of all mice groups were characterized morphologically and biochemically using culture based method. Extensive diversified changes with significant differences in fecal and cecal microbes were observed in mice of groups 3 and 4 compared to groups 2 and 1. Mice fed with GM + HFD (G3) and GM + CD (G4), were characterized by large abundance of *Staphylococcus* sp., *Lactobacillus* sp., *Clostridia* sp., *Bacillus* sp., *Flavobacterium* sp., *Enterobacter* sp., *Escherichia* sp. and *Shigella* sp. While mice fed with HFD (G2) exhibited abundance of *Lactobacillus* sp., *Bifidobacterium* sp., *Bacteroides* sp., and *Enterococcus* sp. compared to mice fed with CD (G1), where increased relative abundance of *Bacteroides* sp., *Prevotella* sp. and fewer *Escherichia* sp. was observed (Fig. 4).

4 Discussion

Globally, obesity is rising as an epidemic and its incidence has more than doubled since 1980\(^7\). This multifaceted and multi-factorial disease is associated with various metabolic and inflammatory diseases including type 2 diabetes, cardiovascular problems, cancer, osteoarthritis, asthma, neurodegeneration and many other similar disease\(^4\). Owing to the existing relationship between gut microbes and intestinal or extra intestinal diseases, science community is paying more attention to understand the underlying mechanisms. According to our knowledge, the current study is first of its kind using cultured gut microbiota of obese patients coupled modified diet (HFD/chow) to investigate their role in inducing obesity in SC mice. Our study revealed that gut microbiota of obese people showed abundance of *Bacteriodes* along with comparatively decrease number of *Firmicutes* as gut microbiomes. Culture independent understanding (16S rRNA) of abundant strains from obese donors comprises of *Lactobacillus acidophilus*, *Lactobacillus reuteri* and *Staphylococcus aureus*. Previously, Million et al.\(^{25}\) in a study reported that gut of obese patients consist of mainly *Lactobacillus* including *L. reuteri*, *L. inlubishi*, *L. acidophilus* and *L. fermentum*. There is close association between the gut microbiota and dietary habits of host. Clark et al.\(^{30}\) reported that mutant mice with induced obesity showed relative abundance of *Bacterioiotes* along with increase proportion *Firmicutes* compared to lean mice. Predominant presence of these two

![Fig. 2](image-url)  
Fig. 2 Effect of diet (HFD/Chow) alone and combined with gut microbes (GM) on body mass index (BMI) of synthetic community (SC) mice (N = 4 mice/group). Mice were given chow diet (group 1), high fat diet (HFD) (group 2), GM + HFD (group 3) and GM + chow diet (group 4). Highly significant increase in BMI was observed in GM + diet supplemented groups (3 and 4), compared to HFD fed (group 2) versus chow diet fed mice (control). Mean values ± SEM were expressed \( (*p < 0.05, **p < 0.001 \) One way ANOVA).
Effect of diet (HFD/Chow) alone and combined with gut microbes (GM) on serum liver parameters of SC mice (N = 4 mice/group). Mice were given chow diet (group 1), high fat diet (HFD) (group 2), GM + HFD (group 3) and GM + chow diet (group 4). After two weeks, mice were sacrificed and serum was isolated from blood. Levels of total cholesterol (TC), triglycerides (TG), high density lipoproteins (HDL), low density lipoproteins (LDL) and very low density lipoproteins (VLDL) were measured. Highly Significant increase in levels of TC, TG and LDL was observed in GM + diet supplemented groups (3 and 4), compared to significant increase in HFD fed (group 2) versus chow diet fed mice (control). Mean values ± SEM were expressed (*p < 0.05, **p < 0.001 One way ANOVA).

Taxonomic distribution of the gut microbiota sample from SC mice fecal and cecal samples. Values represent the average relative abundance across all samples within a given group.
phyla in colon of obese people was reported previously in various different studies. Lower bacterial diversity observed in stool samples of obese patients is in accordance to previous findings reported by Le Chatelier et al. They reported that reduced bacterial diversity is the main factor contributing to excessive weight gain, type 2 diabetes as well as dyslipidaemia. Detrimental transfer in gut microbiota of obese individuals promotes lipogenesis and cause weight gain due to excessive energy supply. We induced the antibiotic depletion of gut microbiota of mice to investigate the effect of GM from obese donors coupled with HFD/chow diet on BMI and liver profile. The fact that BMI of mice fed with chow diet or HFD was lower than that of mice fed with HFD + GM or chow diet + GM is consistent with literature. They demonstrated that obesity-associated gut microbiome had ability to harvest energy from the indigestible dietary polysaccharides. Also, gavage aided colonization of germ free mice with gut microbiota from obese donors showed increase in body fat over 14 days compared to mice colonized with microbiota of lean donors (having chow diet).

GM supplementation to diet (HFD/chow) induced obesity in SC mice, modulated serum liver parameters such as elevated levels of total cholesterol, triglycerides and low density lipoprotein with non significant increase in HDL and VLDL. The importance of gut microbiota for the lipid metabolism of host is undeniable. In this study, firm interplay of obese GM plus diet with host metabolism was observed. We noticed that regardless of diet, GM supplementation form obese patients caused obesity in SC mice. This link between intestinal flora and host metabolism was first established by Turnbaugh et al. They reported that transplantation of intestinal microflora from obese mice resulted in replication of obese phenotype in germ-free mice. The obese microbiota affected host energy and lipid metabolism, causing hypercholesterolemia and hypertriglycerideremia. This is in line with literature where a positive correlation was observed between abundance of obese gut microbiota and plasma cholesterol, LDL and triglycerides. However, we are unsure about factors contributing to hypercholesterolemia (for example, changes in secretion, absorption or de novo synthesis) since we didn’t measure it in cecal contents of any mice. Increased triglycerides observed in this study might be due to alteration in the endogenous hepatic lipid metabolism. Very-low-density lipoproteins (VLDL) are meant to pack triglycerides in the liver. These provide energy in the form of fatty acids to heart and muscles with help of lipoprotein lipases. The low level of VLDL and HDL might be due to over active apolipoprotein H, bounded to their surface and activate lipoprotein lipases (LPL). LPL secretes fatty acids from both VLDL and chylomicrons to provide energy to heart, skeletal muscle and adipose tissue. The lower levels of VLDL observed in this study might correspond the increased chylomicrons which compete with VLDL for LPL. In our study, significant difference was also observed in aforementioned serum parameters in HFD fed compared to chow diet fed mice. Recently, Do et al. assigned six-week-old C57BL/6J mice to receive four distinct dietary regimes: normal diet (ND), HFD, high-glucose diet (HGD), and high-fructose diet (HFrD). After 12 weeks, HFD- and HFrd-fed mice showed significantly higher plasmatic levels of blood glucose, total cholesterol, LDL and endotoxin than those of ND-fed mice.

To elucidate the underlying mechanism, we detected the gut microbiota of two random mice from each of four groups. We observed that GM supplementation had major influence on fecal and cecal bacteria of obese mice compared to control. It resulted in shift of specific microbial diversity and richness in obese mice particularly. Though, using cultivation method it was not possible to test of each and every of individual strain. The dominant genera with GM plus diet (HFD/chow) in SC mice included phyla Firmicutes and Lactobacillus (with prevalent genera of Lactobacillus sp., Staphylococcus sp., Clostridia sp., Bacillus sp. and Enterobacter sp.) compared to shift towards Enterobacteriaceae in HFD/chow diet fed mice. This data thus providing evidence of dysbiosis of gastric microbiota is consistent with literature. Likewise, Zhou et al. showed that fecal microbiota transplantation resulted in decreased Bacteroidetes and increase Firmicutes compared to HFD fed mice. High microbial diversity observed in SC mice having obese GM supplementation with diet (HFD/chow) fed mice is in contrast to the findings published by Sonnenburg et al. However, they used refined low-fiber diet, whereas use of HFD/chow diet with GM supplementation from obese donors is the factor in this study contributing to high diversity of gut microbiome. Despite using limited number of obese strains as GM (65 only in this study) inoculum for mice diet, transmission of obese phenotype was observed irrespective to diet. Similar changes were reported previously by Turnbaugh et al. who provided significance of gut microbiota in transmission of obese phenotype regardless of limited resemblance to donor.

5 Conclusion and Future Perspective
This study provided solid evidence that inoculation of conventional antibiotic-treated mice with gut microbiota from obese donors is quite possible at weaning and resulted in significant alteration of BMI and serum lipid parameters. This is the first study using purified GM of obese humans as feed inoculum to establish SC mice and would have possibility for more pronounced outcomes in other disease models. We have used GM from obese donors, it would be interesting to use GM from lean subjects in obese mice to
investigate its effectiveness against various gastrointestinal infections and associated bowel diseases.

**Author Contributions**

IL designed study, performed research; contributed analytic tools; analyzed data and wrote the manuscript. SN, SE, UH, SR assisted in data analysis and drafting article. NA and RI did critical revision of manuscript. All authors read the manuscript and approved the final draft.

**Conflict of Interests**

All authors have no conflict of interest.

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