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Association of calcium supplementation with fat excretion, changes in body weight, and gut enterobacteriales in obese, and non-obese mice

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ABSTRACT: Obesity is a worldwide epidemiologic syndrome characterized by fat mass accumulation, mainly visceral fat. Current thinking considers a potential role of gut microbiota specially enterobacteriales on the development of obesity and its related comorbidities. Gut microbiota can influence energy extraction from food though alteration of intestinal permeability, lipid metabolism, immune response and endocrine functions and its profile has shown to differ between obese and lean subjects. Consumption of calcium may favor body weight reduction and glycaemic control, but its influence on microbiota is not well understood. This study with mice model focused on the changes of faecal microbiota count in four types of treatment groups and the changes in body weight based on diet and Ca supplement even after making some mice obese. The overall findings show possible correlations of the growth of gram-negative bacteria (*Escherichia coli, Klebsiella pneumoniae and Proteus mirabilis*) and the absence of gram-positive bacteria (*Lactobacillus spp.*) as well as body weight fluctuation under the impact of high fat diet (HFD) and calcium supplementation. Approximately it is observed that HFD influences positively but calcium suppresses the growth of gut enterobacteriales associated with high faecal fat excretion. We conclude that dietary modification with calcium might interfere with gut microbiota, body weight and the beneficiary effects of calcium supplement on obesity can partly be explained by associated high faecal fat excretion.

KEYWORDS: Obesity; gut microbiota; calcium supplementation; faecal fat excretion; body weight reduction.

1. INTRODUCTION

Obesity is one of the world's fastest evolving health challenges both in developed countries and also in developing nations as well. Childhood and adolescent obesity are associated with a number of medical complications: hypertension, type 2 diabetes, coronary heart disease, stroke and several types of cancer [1–3]. The etiology of this epidemic is multifactorial, including excessive calorie intake [4], a sedentary lifestyle [5], genetics [6], as well as socioeconomic elements, such as lack of access to unprocessed food. The high fat and sugar content in processed foods are implicated in increased adiposity [7]. Moreover, gut microbiota has been recorded to have implication in obesity. Transplanting microbiota from obese animal to germ-free mice resulted in a greater increase in total body fat compared with transplanting microbiota from lean animals, highlighting the contributory role of microbiota to obesity [8]. Microorganisms promote more efficient extraction or storage of energy from indigestible common polysaccharides in our diet for their capability to adaptively deploy a large

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array of glycoside hydrolases and polysaccharide lysases that we humans do not encode in our genome [9, 10]. Consumption of a high-fat meal in both animals and human results in significant increases in endotoxin (lipopolysaccharide (LPS)) concentrations due to increased intestinal permeability and changes in the gut microbiota composition [11, 12].

Furthermore, the beneficial effects of Ca ingestion could be associated with intestinal microbiota modulation and with increased integrity of the intestinal mucosa. High-calcium diets seem to change the intestinal environment through : (I) Increasing gastric secretion leading to increased gastric pH and reduced number of viable bacteria; (II) Causing bile acid and fatty acid precipitation, increasing colonic pH and reducing cytotoxic components (especially Non-esterified fatty acid (NEFA) and ionized secondary bile acids) that damage the epithelial cells; (III) Increasing glucagon-like peptide-2 (GLP-2) secretion, which has a trophic effect on intestinal mucosa and reduces gene expression of tight junctions (occludin and zonula occludens-1). These mechanisms may reduce bacterial and lipopolysaccharide (LPS) translocation, by bacterial fermentation and intestinal microbiota modulation, leading to a highly selective and controlled intestinal permeability [13]. The composition of the gut microbiota is constantly being changed by many factors such as diet, disease state, medications specially antibiotics as well as host genetics etc. affecting the health and well-being of the host [14].

Recently it has been hypothesized that salt may play a critical role in the obesity epidemic. Observational studies have found that dietary calcium intake is inversely related to body weight and body fat mass. The interaction between dietary Ca and FA in the gut forms insoluble Ca-FA soaps, which in turn increases faecal fat excretion indicating pancreatic exocrine insufficiency and reduces dietary energy [15]. It was estimated that increasing the calcium intake results in an increase in faecal fat excretion to an extent that could be relevant for prevention of weight (re-) gain. Thus, a primary objective of our study was to determine the effect of dietary salt on adolescent male & female fed either a normal or high fat diet relating to the changes of microbiota growth.

2.RESULTS

2.1 Bacterial growth and count

This study was aim to isolate and identification of various pathogenic bacteria from mice dry faeces. All of the samples that were collected from different treatment group of mice from individual boxes were processed through several steps. Dilution, selective plating, biochemical tests, gram staining had been applied for isolation and identification of bacteria in collected samples. There were no gram-positive bacteria that identified in plates but all were gram negative. A total of 4 isolates (gram negative*: Escherichia coli (E. coli), Klebsiella pneumoniae and Proteus mirabilis*) and gram positive: *Lactobacillus spp.*) were selected from all samples enrolled in the study and two different media namely MacConkey agar (for *E. coli, Klebsiella pneumoniae and Proteus mirabilis*) and MRS Lactobacillus (for *Lactobacillus spp.*) were used. The samples contained 3 different isolates markedly that were confirmed in Figure 1by biochemical tests as *E. coli, Klebsiella pneumoniae and Proteus mirabilis* showed in Table 1. Comparative microbial growth of a certain week from faeces of non-obese group has been represented in Figure 2. Colony count of these medias in CFU (Colony Forming Unit)/mg unit is considered as total bacterial count of the sample.

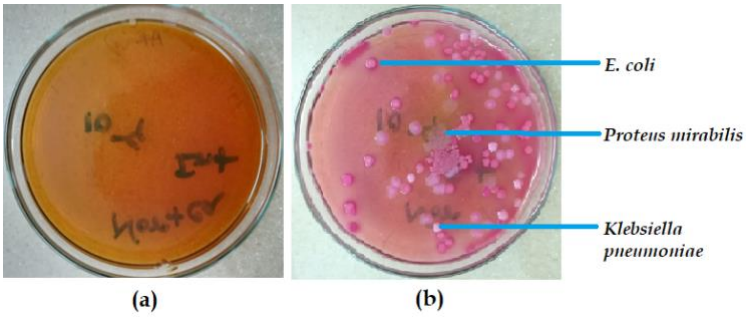

Figure 1.(a) No growth of *Lactobacillus spp.* on MRS Lactobacillus media plate**, (b)** Culture of *E. coli, Klebsiella pneumoniae and Proteus mirabilis*on MacConkey Media plate.

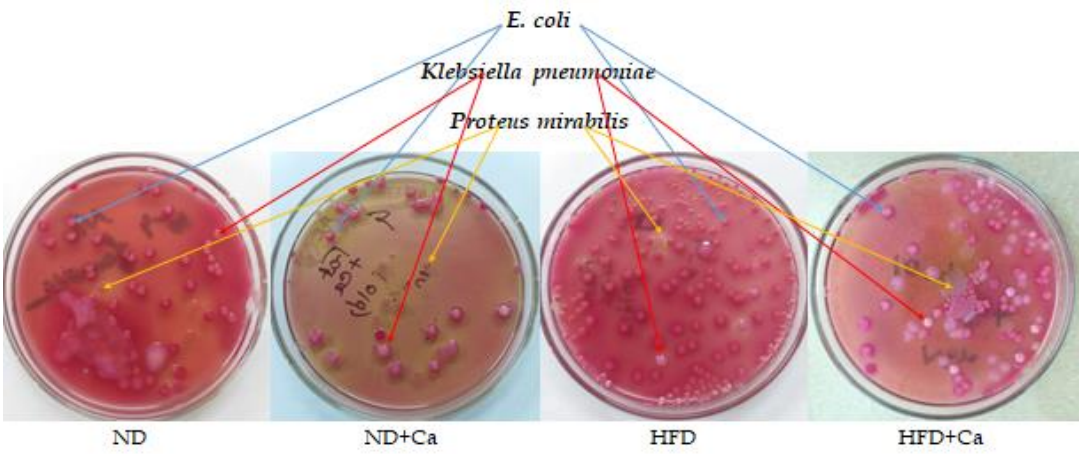

Figure 2. A comparative plate representation of a certain week of Non-obese group (10^{-4} dilutions).

Table 1. Biochemical test results of all samples.

Growth on		Gram Staining	Biochemical tests				Suspected Organisms
MacConkey	**MRS Lactobacilius**		**MIU**	**SCA**	**TSI**	**Oxidase**	
+	-	-	+/+/-	-	A/-/+	-	*Escherichia coli*
+	-	-	-/-/-	+	A/+/-	-	*Klebisella pneumoniae*
+	**-**	-	+/+/+	-	A/+/+	-	*Proteus mirabilis*

*Here, A= Acid (Yellow) reaction.

2.2. Isolates count from weekly based group wise faecal samples

The changing count in faecal microbiota growth among four types of treatment groups based on diet and calcium supplement even after making some mice obese show some indications in this study. Considering the trendline of changes in Figure 3, it can be assumed that *E. coli* increases in HFD, HFD+Ca comparing ND group where in ND+Ca, *E. coli* slightly decreases than ND. Besides, *E coli* decreases in HFD+Ca comparing HFD group. On the other side, Figure 4 apparently indicates that *Klebsiella pneumoniae* increases in HFD, HFD+Ca comparing ND group where in ND+Ca no changes in count from initial abundance compared to ND. But *Klebsiella pneumoniae* decreases in HFD+Ca comparing HFD group. On the contrary, Figure 5 seemingly shows that *Proteus mirabilis* increases in ND but in ND+Ca no increase from initial count. *Proteus mirabilis* decreases in HFD, HFD+Ca successively compared to ND group.

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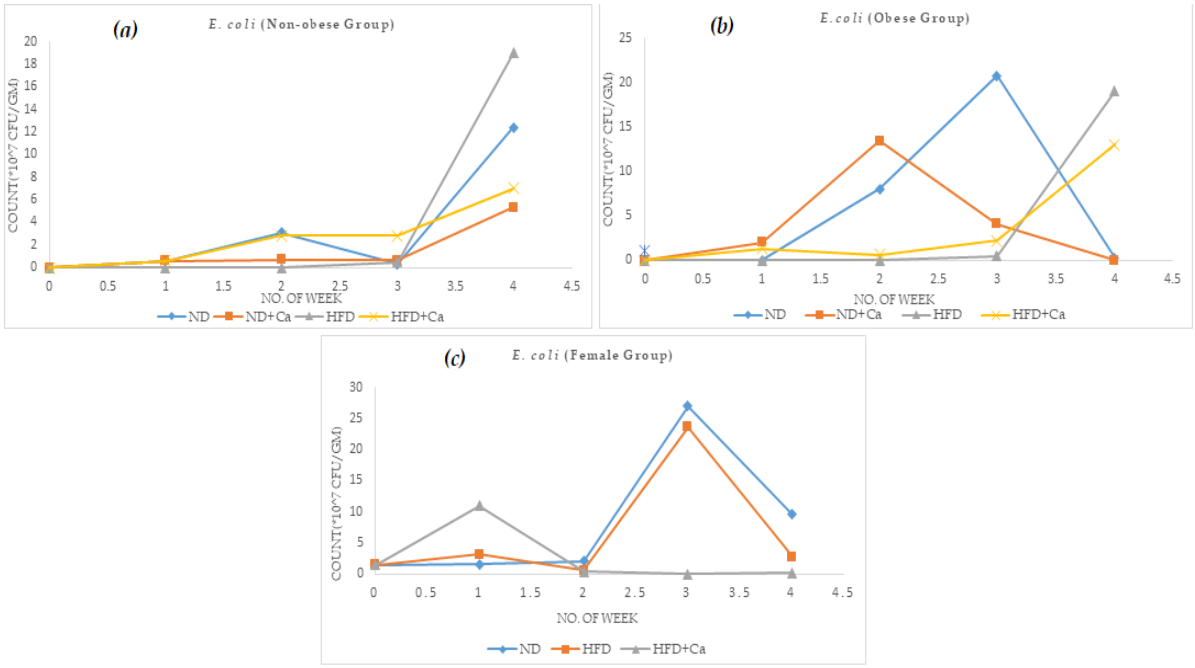

Figure 3. *E. coli* in MacConkey Agar during last 4 weeks' treatment period on (a) Non-obese mice, (b) Obese mice and (c) Female mice.

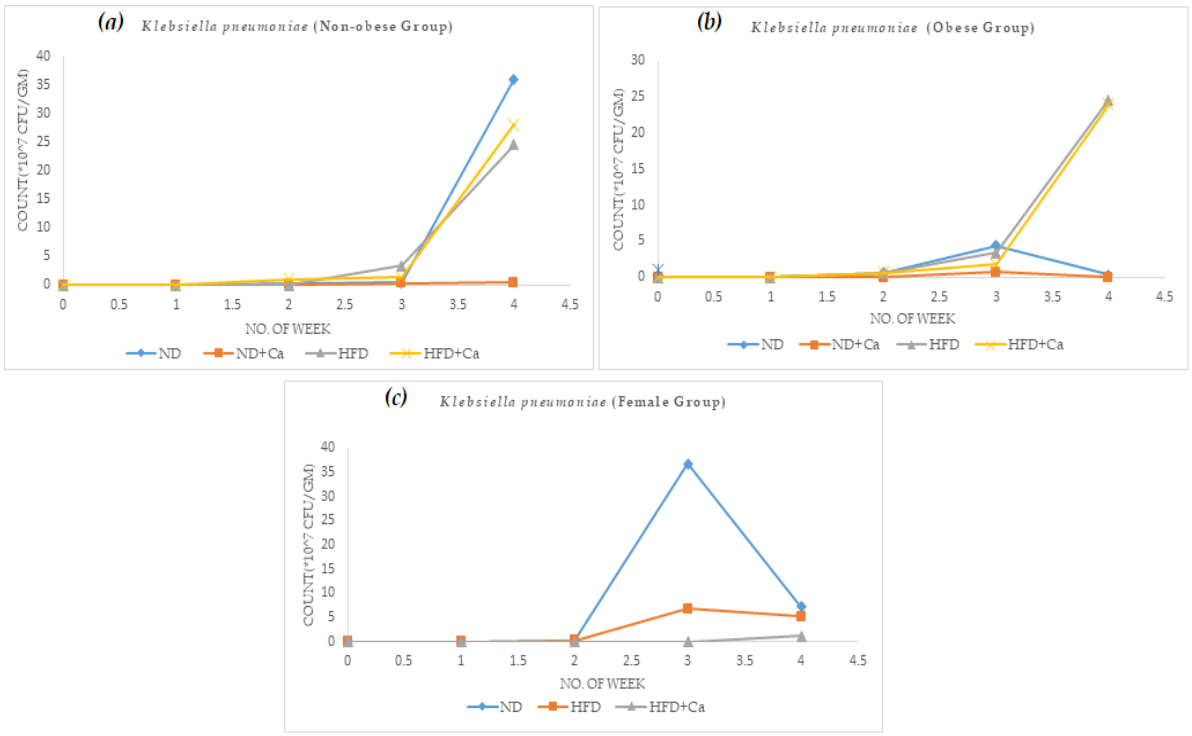

Figure 4. *Klebsiella pneumoniae* in MacConkey Agar during last 4 weeks' treatment period on (a) Non-obese mice, (b) Obese mice and (c) Female mice.

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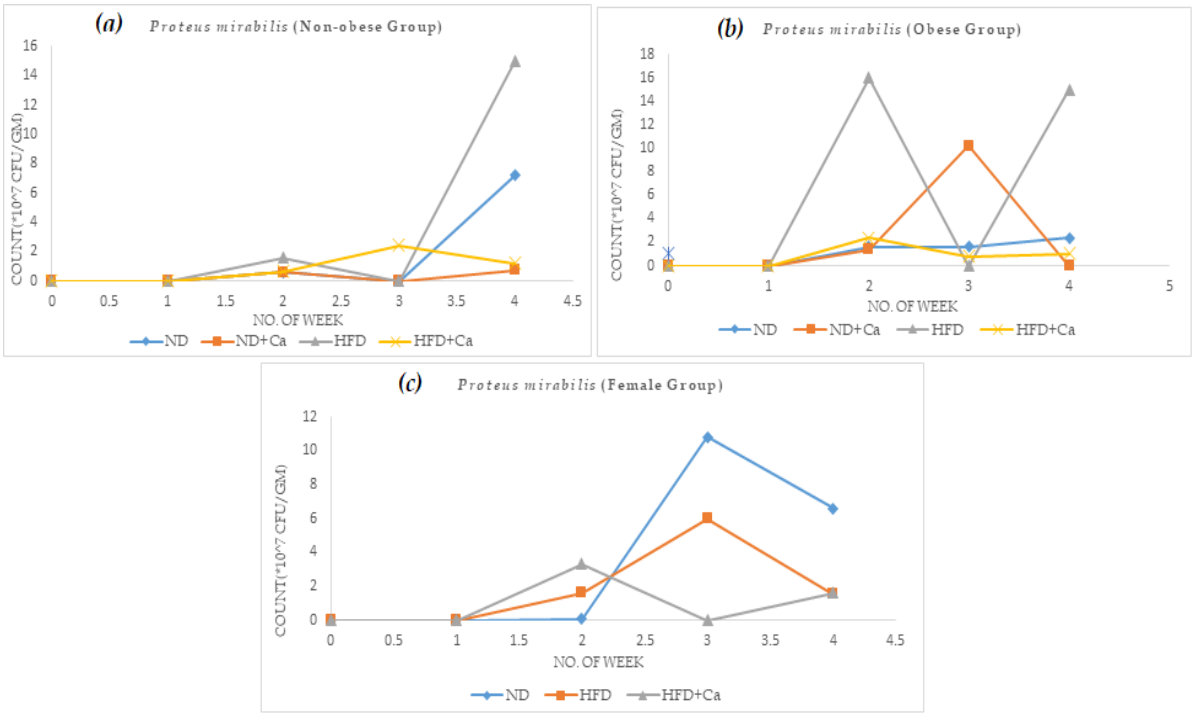

Figure 5. *Proteus mirabilis* in MacConkey Agar during last 4 weeks' treatment period on (a) Non-obese mice, (b) Obese mice and (c) Female mice.

2.3. Body weight gain percentage of mice after 4 weeks' treatment period

The results of the research in Figure 6 show that there is a decrease in body weight in calcium diet groups (ND+Ca and HFD+Ca) compared to ND and HFD group in case of Non-obese, Obese and Female mice.

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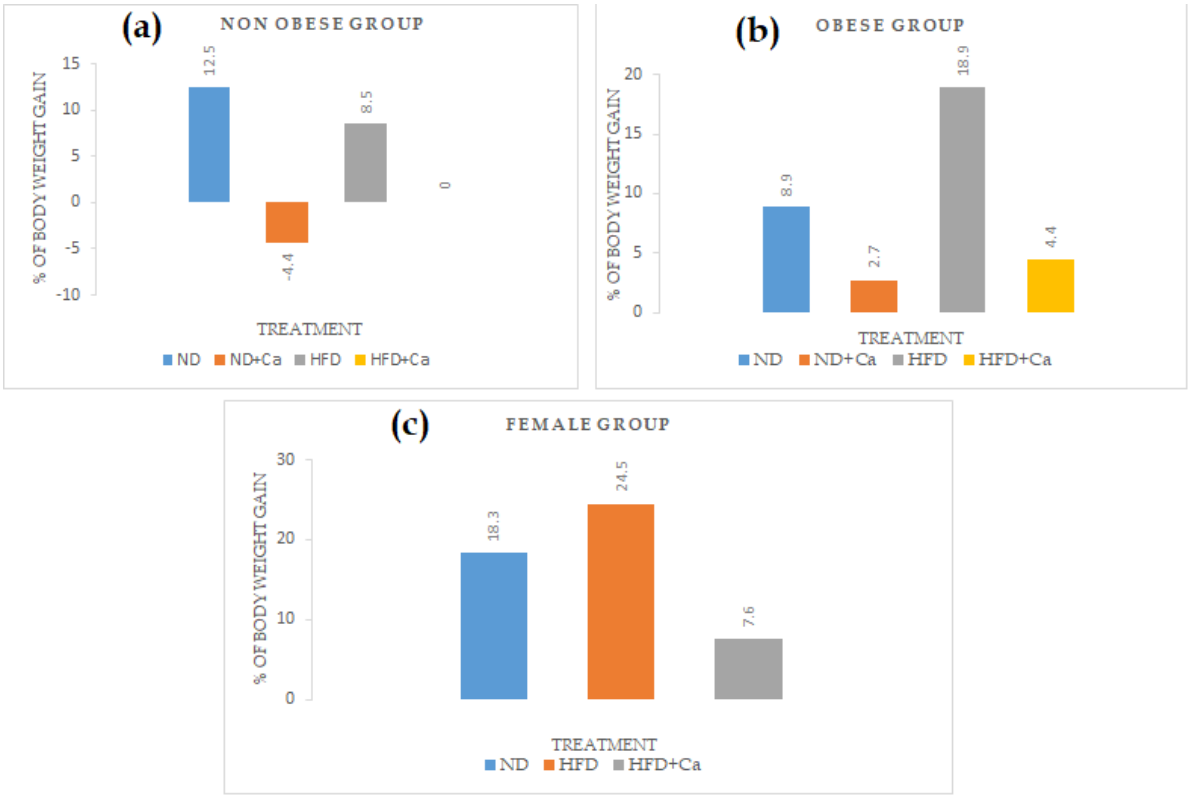

Figure 6. Percentage of body weight gain of mice after 4 weeks' treatment period of (a) Non-obeseGroup, (b) ObeseGroup and (c) Female Group.

2.4. Fat excretion through faeces

By deep observation of color darkness in Formalin-Dye and Perchloric Acid –Dye color effects, it has been found in summary that fat is comparatively more excreted through faeces of HFD group than that of ND. As the darkness of color effect of the test sample liquid mixtures is higher successively from ND to ND+Ca to HFD to HFD +Ca presented in Figure 7 and Figure 8, these tests prove that the impact of Ca treatment also causes more fat excretion than the controlled sample.

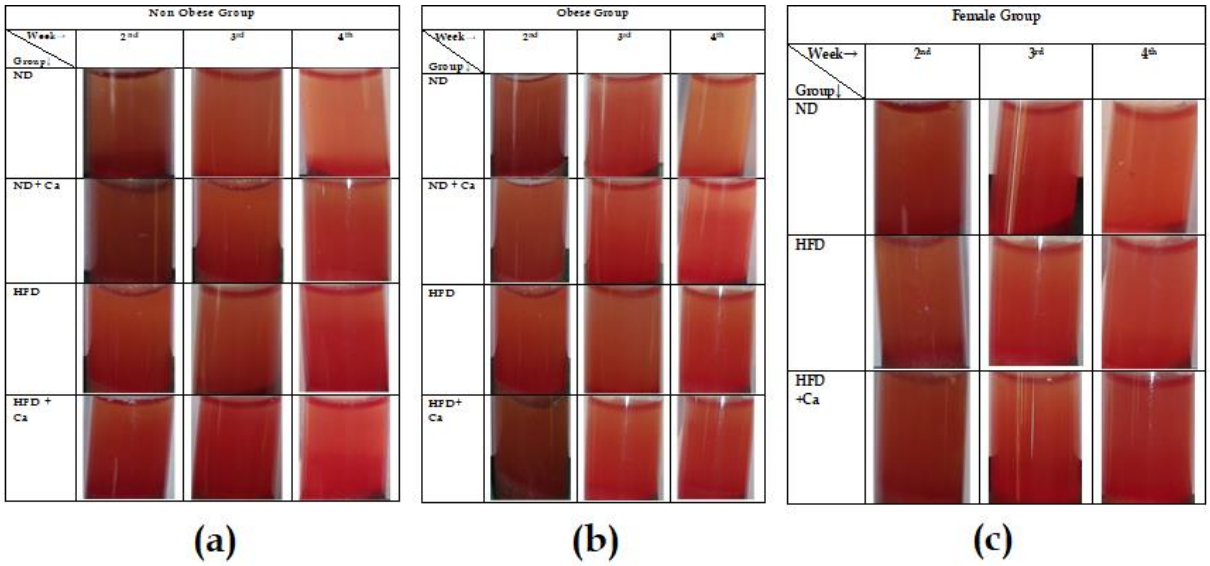

Figure 7. Formalin-Dye Color Effects (a) Non-obese Group, (b) Obese Group and (c) Female Group respectively.

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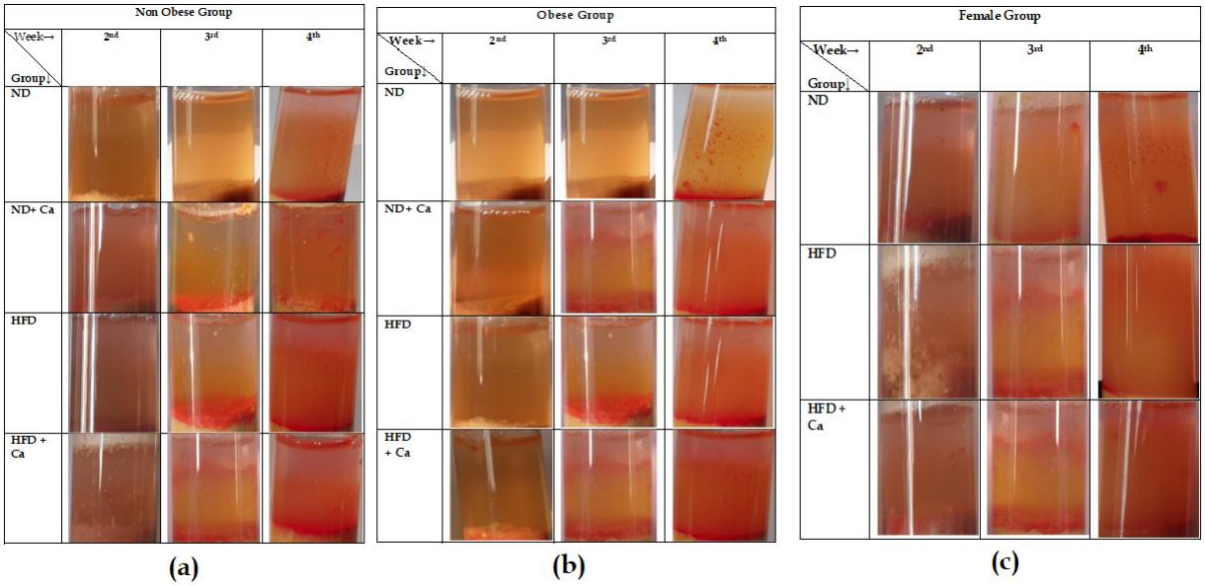

Figure 8. Perchloric Acid-Dye Color Effect (a) Non-obese Group, (b) Obese Group and (c) Female Group respectively.

3. DISCUSSION

Scientific evidence supports that dietary pattern directly changes the gut microbiota profile resulting in different gut microbiota composition and activity that can contribute to amelioration of obesity [16]. The gut microbiota may also influence the development of conditions characterized by low-level inflammation including obesity through systemic exposure to bacterial lipopolysaccharide derived from the intestinal microbiota suggesting that modification of the gut microbiota may be a relevant therapeutic avenue for obesity and other metabolic disorders [17]. One potential strategy to reduce obesity is to consume calcium, which has been implicated to be involved in reducing bodyweight/fat [18]. It has been demonstrated that high-calcium diets appear to positively affect gut microbiota composition [13]. In humans, it has been shown that supplementation of calcium decreases fat absorption and increases the fecal excretion of insoluble calcium soaps with fatty acids [15]. Similarly, a short-term increase in dietary calcium intake promoted fecal fat and energy excretion [19].

This study focused on the changes of faecal microbiota count in four types of treatment groups and the changes in body weight based on diet and calcium supplement even after making some mice obese. It is observed that HFD significantly increases both the body weight and faecal fat excretion. Besides treatment with calcium salt significantly decreases the body weight and promotes more faecal fat excretion that was also proved by the color effect testsin another research [20]. Unexpectedly we found no rigid trendline to conclude the overall findings of the changes infaecal microbiota count. There may be the lack of a robust protocol to conduct the research for clarification of the actual outcome among the growth of *E. coli*, *Klebsiella pneumoniae and Proteus mirabilis*. From the apparent point of view, here *E. coli* bacteria shows differences between the four treatment groups among these Non-obese, Obese and Female mice possibly reflecting the impact of obesity and calcium resulting to increase for obesity but decrease for calcium treatment. In mice feaces, a positive correlation has been observed between the growth of *E. coli* and that of *Klebsiella pneumoniae*, but negative relationship with *Proteus mirabilis* growth during the treatment with high fat diet. It can be assumed that a treatment with calcium salt may suppress the growth of *E. coli*, *Klebsiella pneumoniae and Proteus mirabilis* though the growth of first two of three isolates is increased and *Proteus mirabilis* growth is suppressed with HFD. Although the average age of the Non-obese, Obese mice was higher than that of the Non-obese female mice in the study, we found no correlations between any bacteria and subject age indicating that the differences in gut microbiota among obese, non-obese and female mice were not attributable to differences in the age of the mice. As per our observations, mice fed a high fat diet gained more weight than their normal diet fed counterparts but when salt was added to

the high fat diet, the high fat-induced weight gain was minimized. Thus, the current evidence demonstrates the anti-obesity effects of calcium and suggests the potential application of dietary calcium for prevention of obesity.

4. CONCLUSION

The contribution of dietary calcium modulation to the control of obesity is uncertain. To make insight clear, we demonstrated a significant association among HFD, changes in body weight, calcium supplementation and gut microbiota composition in the mice model study. We identified three isolates that bear witness to different growth tendency in these treatment groups. In addition, we found that there was a correlation between the relative abundance of enterobacteriales' isolates and obesity. These observations support the notion that an interaction exists between diet salt content, gut microbial compositions. Moreover, human clinical trials are needed to explore the potential of dietary calcium or calcium salt supplementation in the modulation of microbiota and intestinal barrier integrity and to determine the applicability of relatively simple dietary interventions to the treatment of chronic diseases. Further research is required with another established protocol to define the supplementation period, the dose and the type of calcium supplement (milk or salt) that is more effective in healthy and obese subjects. As calcium interacts with other components of the diet, these interactions should also be considered in future research.

5. MATERIALS AND METHODS

5.1. Chemicals and reagents

Calcium Carbonate ($CaCO_3$) from Merck India,MacConkey agar media from Becton Dickinson (BD) USA, MRS Lactobacillus media from BD USA, EMB (Eosine –methylene blue) from Himedia India, Sodium Chloride (NaCl) from Merck India, Citrate (Simmon's Citrate Agar) reagent from BD USA, MIU (motality, indole, urease) reagent from Himedia India, oxidase reagent from LOBA Cheme India, Triple Sugar Iron (TSI) reagent from Himedia India, Perchloric Acid from DAEJUNG Korea , Isopropyl Alcohol from ScharlauSpain, Formalin from Merck India, Sudan II dye from Difco Lab UK were used in this study.

5.2. Experimental animals

The study protocol was designed with 50 male-female Swiss Albino mice (35 male & 15 female) procured from JU, 5 weeks' old (weight: 22–25 g)which were housed in regular cages (5 mice per cage as a single group) in an animal room at 22°C. Fresh and clean normal water was supplied ad libitum through the specific nipple. The cages of the mice were cleaned regularly, and water and food were supplied twice daily. Acclimatization was done for 7 days before conducting the experiments. All animal experiments were conducted at the Department of Pharmacy, Noakhali Science and Technology University, Bangladesh.

5.3 Study design

Firstly 35 male mice were grouped and maintained as groups based on dietary modification in Figure 9 where the initial 4 groups (Non-obese) of male mice were treated for 10 weeks and the final 4 groups (Obese: separated and grouped at 6^{th} weeks from initial 20 male Non-obese mice) for 4 weeks separately. The study protocol was approved by the ethics committee of Noakhali Science and Technology University (Ref: NSTU/SCI/EC/2022/87).

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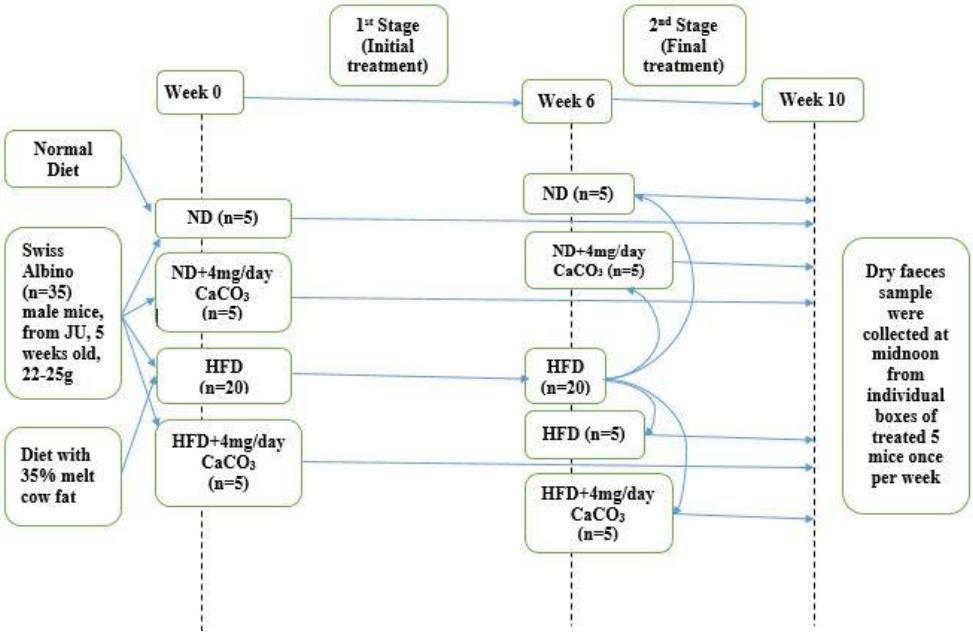

Figure 9. Study design of male mice.

Besides this experiment, another 15 newly collected female (Non-obese) mice were treated in 3 groups for 4 weeks as in Figure 10.

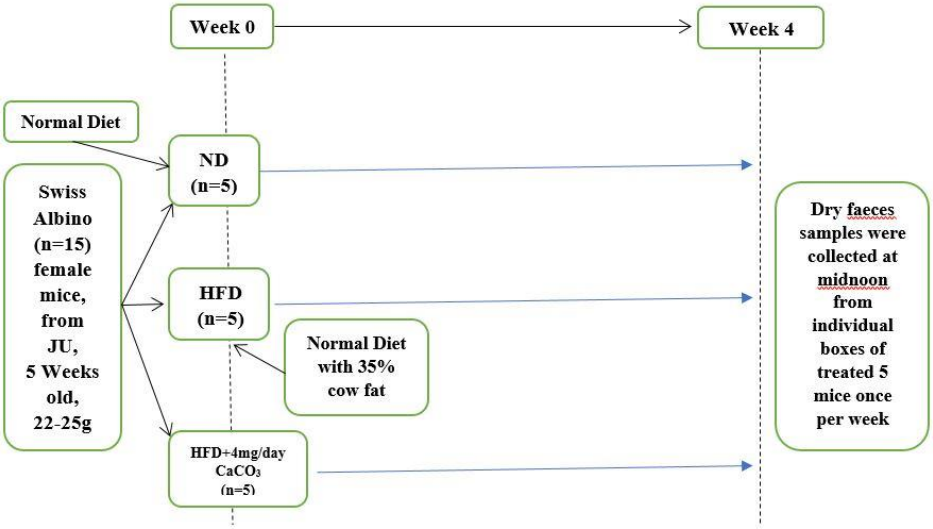

Figure 10. Study design of female mice.

5.4. Collection of samples

During this study, dry faeces samples were collected at midnoon from individual boxes of treated 5 mice once per weekbased on study design. Faeces of 5 mice of one individual box considered as unique sample as faeces amount of a single mouse was not sufficient for experiment. For this, the used boxes were cleaned aseptically on the previous evening and the mice were transferred into them group wise for faeces collection. All samples were collected aseptically using sterile gloves and test tubes to prevent any other contamination.

5.5. Processing and microbial analysis of representative isolates

Solid samples were crushed by sterile mortar and pestle. Then 1g. of sample weighed for homogenization in 9ml sterile normal saline (0.9% NaCl solution). Then the samples were homogenized with vortex machine.

From those homogenized faeces samples (10^{-1}), 10-fold serial dilutions were made for 10^{-2}, 10^{-3}, 10^{-4}, and, 10^{-5} using sterile normal saline [21,22].

5.6. Isolation of bacteria using selective media

After sample preparation, 100 µl from each dilution of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and, 10^{-5} were plated on MacConkey agar media and MRS Lactobacillus media as selective media was used to isolate or identify selective organism. MacConkey agar media is selective for gram negative bacteria where MRS Lactobacillus for gram-positive *Lactobacillus* spp. present in that particular dilution of the sample. All the test samples were plated individually by following spread plate technique. Then the plates were incubated for 24 hours at 37°C [23]. Plates were removed when colonies had developed and then the colonies were counted in the sample dilutions which contains 3-30 colonies per 10 µl drop. Viable cell counts are expressed as colony forming units (CFU)/surface area.

CFU per gm. = Average number of colonies for a dilution x 10 x dilution factor [22].

5.7. Maintenance of the culture

The pure colonies on MacConkey agar media were preserved at 4°C by repeated streaking for biochemical tests for confirmation.

5.8. Microscopic observation by gram staining

A differential staining method was used for distinguished the bacteria into two groups: gram positive and gram negative. All the isolates were examined microscopically after gram staining to identify whether the isolates were gram (+) or gram (-) and to observe their arrangements [24].

5.9. Biochemical characterization

The identification of colonies of the isolates were done by performing various biochemical tests. For Gram (+) ve bacteria and Gram (-) ve bacteria, Citrate utilization test, MIU (motality, indole, urease) test, oxidase test, Triple Sugar Iron (TSI) (acid slant/acid butt/gas) test were performed. All the tests were performed according to the standard protocol [25].

5.10. Fecal fat excretion analysis

A recent study reported that salt impacts the digestive efficiency of lipids [20]. Thus, we performed 2 independent assays of faecal lipid content as described below:

5.10.1 Perchloric acid-dye test

(I) Dry faeces (approx. 1 g) were collected from individual box of treated 5 mice once per week across the duration of the experiment. (II) Lipids were extracted by pulverization with a mortar and pestle. (III) 500 mg dust was vortexed for 5 minutes with 5 ml 1 N Perchloric acid in a test tube. (IV) Mixture was allowed for sedimentation for 10 minutes. (V) 1 ml Sudan II dye solution (Previously prepared by 0.25 g Sudan II Dye powder with 25 ml IPA to be up to 100 ml volume) was added to the settled liquid. (VI) The color effect of the tubes was captured after 5 minutes [20].

5.10.2. Formalin-dye test

(I) Dry faeces (approx. 1 g) were collected from individual box of treated 5 mice once per week across the duration of the experiment. (II) Lipids were extracted by pulverization with a mortar and pestle. (III) 100 mg dust was vortexed for 5 minutes with 2 ml formalin in a test tube. (IV) Mixture was allowed for sedimentation for 10 minutes. (V) The upper clear liquid portion was allowed for decantation and sediment portion of bottom was kept in the origin tubes. (VI) 1 ml Sudan II dye solution (Previously prepared by 0.25 gm Sudan II dye powder with 25 ml IPA to be up to 100 ml volume) was added to that bottom portion. (VII) The color effect of the tubes was captured after 5 minutes [20].

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Conflict of interest statement: The authors declared no conflict of interest.
Ethics committee approval: The study protocol was approved by the ethics committee of Noakhali Science and Technology University (Ref: NSTU/SCI/EC/2022/87)

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