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The Effect of Macronutrient Restrictions on Gut Microbiome and Biochemical Parameters of Wistar Albino Rats

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Abstract

Macronutrients serve as a source of energy for both gut microbiota and its host. An increase or decrease in macronutrients can either increase or decrease the composition of gut microbiota, leading to gut dysbiosis which has been implicated in many diseases state including non-communicable diseases. To achieve this, seven diets were formulated by restricting 60% of each macronutrient. These diets were fed on 42 albino rats (Wistar), divided into 7 groups of 6 rats each. Group 1 was fed on a normal laboratory chow diet (ND), group 2 received a fat-restricted diet (FRD), group 3 received a protein-restricted diet, (PFD), group 4 received a carbohydrate-restricted diet (CRD), group 5 received a protein and fat-restricted diet (PFRD), group 6 received a carbohydrate and fat-restricted diet (CFRD) and group 7 received a carbohydrate and protein-restricted diet (CPRD). Feed and water intake were given ad libitum and daily weight and food intake were recorded. The experiment went on for 4 weeks after which animals were sacrificed and intestinal content and blood were collected for analysis (gut microbial composition, glucose, insulin levels, serum lipid, and enzyme). Compared to the control group results showed a decrease in Bacteroides (40.50 - 14.00 CFU), HDL (68.20 - 40.40 mg/dl), and AST (66.62 - 64.74 U/L) in FRD. An increase in AST (66.6 - 69.43 U/L), Bifidobacterial (59.50 - 92.00 CFU) and decreased Bacteroides (40.5 - 19.5 CFU) for PRD was also recorded. CRD reduced Lactobacillus (73 - 33.5 CFU), total bacterial count (129 - 48 CFU), HDL (68.2 - 30.8 mg/dl), and cholesterol (121.44 - 88.65 mg/dl) whereas intestinal composition of *E. coli* (30.5 - 51.5 CFU) increased. PFRD increased Lactobacillus (73.00 - 102.5 CFU), Bifidobacterial (59.5 - 100 CFU), HDL (68.2 - 74.7 mg/dl), and Triglyceride (111.67 -

146.67 mg/dl) concentration. Meanwhile, a reduction in Bifidobacterial (59.5 - 41.5 CFU), and an increasing of AST (66.62 - 70.30 U/l) were recorded for CFRD. However, Bacteroides (40.5 69.5 CFU), LDL (30.95 - 41.98 mg/dl) increased and Bifidobacterial (59.5 - 38.00 CFU) and HDL (68.2 - 53.5 mg/dl) decreased for CPRD. This work, therefore, concludes that macronutrient restriction causes significant changes in serum marker and enzyme profile, and gut microbial composition which can cause gut dysbiosis and later on could expose the host to inflammatory diseases in the long run.

Keywords

Diets, Dysbiosis, Gut Microbiome, Lipid Profile, Serum Enzymes, Non-Communicable Disease, Gut Microbiota, Gut Dysbiosis, Restricted Diet

1. Introduction

Macronutrients are the main source of energy for both humans and microorganisms found in their gut. These microorganisms are termed gut microbiota. Humans acquire this gut microbiota during and after birth, in infancy and toddler years [1]. Several factors affect the gut microbiota composition. These include diet, host genetics, use of antibiotics, diseases, aging, and environmental factors [2]. The gut microbiota plays several functions such as harvesting energy from food, improving gut transit and function, protecting against pathogens, producing vitamins and hormones, and other metabolites. An imbalance in the gut microbiota ecosystem is termed microbiota dysbiosis which is caused by several factors including diet [3]. Gut microbiota dysbiosis has been observed in many inflammatory diseases of the gastrointestinal tract (GIT) and organs linked to the track, either metabolically or immunologically. Interestingly, diet appears to be the most important determinant of gut microbial composition [4]. Short-term dietary interventions in healthy humans lead to significant and rapid alterations in the composition of the intestinal microbiota, but the magnitude of these effects is modest, relative to inter-subject variability [5].

A recent study shows that macronutrient composition and feeding paradigm result in a significant alteration in gut microbiota composition. These changes may improve physiological and cognitive outcomes following dietary implementation for both healthy and diseased humans [6]. Another study suggests that dietary fat has a beneficial role in serum lipid levels by lowering serum triglyceride levels and TC: HDL-C ratio, whereas carbohydrate intake was adversely associated with lipid profile by decreasing serum HDL-C concentrations [7]. According to Mutner *et al.* restricting protein decreases the intake of saturated fatty acid, fat cholesterol, and insulin resistance [8].

Given the worldwide epidemic of diet-related chronic diseases, evidence-based dietary recommendations are fundamentally important for health promotion. Despite the importance of the human gut microbiota for the physiological effects

of diet and chronic disease etiology, national dietary guidelines around the world are just beginning to capitalize on scientific breakthroughs in the microbiome field [8] [9]. It will therefore be time to rethinking healthy eating in light of the gut microbiome. Nevertheless, the available evidence is strongly in support of an important role of the gut microbiome in the effects of diet, emphasizing that a mechanistic understanding of diet-microbiome interactions can inform nutrition controversies and advance the development of healthier diets. A gut microbiota-targeted dietary intervention for amelioration of chronic inflammation underlying metabolic syndrome can be derived from gut studies and will present a variety of considerations including both practical recommendations that can be immediately incorporated into studies and aspirational recommendations that will require greater effort for implementation. It is difficult to determine which foods are the most promising candidates for intervention trials. Studies with strong dietary data collection methods will therefore play an important role in identifying potential diet-derived bioactive compounds and their food sources that can be investigated more closely with well-designed interventional studies [9].

Non-communicable diseases (NCD) such as cancer cardiovascular disease and diabetes are increasingly becoming the main cause of death in sub-Saharan Africa where these diseases were responsible for 37% of deaths in 2019 rising from 24% in 2000 [WHO]. In Cameroon, statistics show that cardiovascular diseases account for most deaths [9]. The natural homeostasis of the gut microbial community changes during many disease pathologies including obesity, metabolic syndrome, diabetes, cardiovascular disease, and celiac disease. In many cases, there is evidence implicating gut dysbiosis to these diseases. The evolution of diet over time caused by factors such as changes in food availability, food prices, and level of income, has forced individuals to replace traditional plant-based diets to diets high in sugars, animal fat, and food additives which are largely associated with gut dysbiosis [10]. The growing use of pesticides in agriculture can cause accidental chemical consumption such as lingering pesticides on washed fruits which can cause gut dysbiosis [11]. The development of new medications such as antibiotics affects the gut microbiota leading to rapid and diminishing levels of gut flora that leads to gut dysbiosis [12]. What effect does restricting macronutrients have on gut microbiota, lipid profile, and serum enzyme activities? This work aimed at evaluating the effect of macronutrient restricted diet on the gut microbiota load and biochemical parameters of non-communicable disease in Wistar rats.

2. Materials and Methods

2.1. Study Design

This is a laboratory-based study, conducted on different formulated macronutrient restricted diets using Wistar albino rats as the experimental model for a period of 4 weeks.

2.2. Ethical Clearance

Ethical clearance approval was obtained from the University of Buea-Institution for Animal Care and Use Committee (UB-IACUC) with permit Number: UB-IACUC N˚ 02/2023.

2.3. Feed Formulation

Ingredients for the formulation of various diets were bought from the local market located at Great Soppo, Buea South-west Region. These include: Soybean oil, corn-starch, Fish meal, corn fiber, Mineral mix (AIN-93G-MX), Vitamin Mix (AIN-93VMX). The ingredients were transported to the teaching laboratory of the Department of Biochemistry and Molecular Biology, University of Buea, where the formulation was made. The appropriate weights of various ingredients were measured using an electronic balance according to Table 1. The ingredients were mixed appropriately in a large bowl. After obtaining a homogenous mixture, each diet was sealed using an airtight zip lock plastic and kept in a dry clean container ready for use. The seven diets were isocaloric (3677 kcal), the primary distinguishing feature being protein, fat and carbohydrate content. The required macronutrient distribution in the seven diet groups was achieved by replacing a proportion of energy derived from carbohydrate (mainly corn starch) with fats (mainly soybean oil) and protein (mainly fishmeal). Same distribution was done for fat and protein. Each diet was formulated per kg.

2.4. Study Design

This is a laboratory-based study, conducted on different formulated macronutrient restricted diets using Wistar albino rat as the experimental model for a period of 4 weeks.

Table 1. Composition of diet in g/1000 for the different groups.

GROUP Ingredient	1	2	3	4	5	6	7
	ND	FRD	PRD	CRD	PFRD	CFRD	CPRD
Cornstarch (g)	529	589.3	609.7	211.6	709.4	211.6	211.6
Fish meal (g)	200	222.8	80	441.1	80	671.9	80
Sugar(g)	100	111.4	115.2	40	134.1	40	40
Soybean oil (g)	70	28	80.7	130.5	28	28	291.1
Fiber (g)	56	56	56	56	56	56	56
Mineral (g)	35	35	35	35	35	35	35
Vitamin (g)	10	10	10	10	10	10	10

ND: Normal diet, FRD: Fat restricted diet, CRD: Carbohydrate restricted diet, PRD: protein restricted diet, PFRD: Protein and fat restricted diet, CFRD: Carbohydrate and fat restricted diet, CPRD: Carbohydrate and protein restricted g: grams. Source: adapted from [14].

2.5. Experimental Animals

A total of forty-two (21 males and 21 females) albino rats (Weight: 130 - 180 g) used for this study were purchased from animal house of the Department of Animal Biology at the University of Yaounde. They were housed in the animal house at the University of Buea and maintained under standard conditions of temperature (22˚C) and 12 h light/dark cycle. They were acclimatized to laboratory conditions for a period of one week and all rats had access to normal laboratory chow feed (22% protein, 62 % carbohydrate, and 16 % fat) and water ad libitum during the experimental period.

2.6. Experimental Design

The experiment was carried out using the method described by Liu *et al.* [13]. The rats were randomly distributed into seven (7) groups of six (3 males and 3 females per group) animals each. They had access to their respective diet (s) and water ad libitum. During the experiment, their daily feed intake and body weight were evaluated and the mean was calculated. The experiment lasted for 28 days.

2.7. Weight and Food Intake

Food intake and weight were measured daily during the experiment. This was done by briefly removing the rats from their cages and weighing them, and the amount of food remaining (including any on the bottom of the cages or any that had spilled onto plastic sheets placed under each cage), was recorded. Intake was calculated as the weight (in grams) of food provided minus food left in the cage. Individual weights of all rats were measured using an electronic balance.

2.8. Collection, Processing and Samples for Analysis

2.8.1. The Oral Glucose Tolerance Test

On the 28th day of the experiment, the animals were fasted overnight after which the blood glucose was measured using a glucometer and denoted as fasting glucose (baseline glucose at t0) before administering glucose (2 g/kg). After that further measurements were made at a regular interval of 15, 30, 60 and 120 minutes.

2.8.2. Insulin Levels

Insulin levels were measured using fluorecare insulin kit according to the manufacturer instructions. The principle of the immune-chromatographic assay is used to detect the concentration of insulin in serum or plasma by double antibody sandwich method. Insulin in the specimens is combined with the fluorescent antibody on the bonding pad, and an insulin antibody complex is formed. Because of the effect of the chromatography, Insulin-antibody complex will be diffused along the nitrocellulose membrane. In the test line (T) region, the insulin-antibody complex is to be combined with coated antibody in the dictation area. The higher the concentration of insulin, the more the aggregation of the complex on the detection line the deeper the color of the fluorescent band under

the specific wavelength.

2.8.3. Collection of Blood and Serum Preparation

On the 29th day, blood was collected from the heart of the overnight fasted rats under chloroform anesthesia. The blood collected was allowed to clot and centrifuged at 4000 rpm for 15 min at room temperature to obtain the serum. The blood serum was kept in a refrigerator at 0˚C - 4˚C until it was used.

2.8.4. Collection of Intestinal Contents

In a sterile environment, the feces found in the intestine were gently brushed off the wall of the intestine, from the jejunum to the rectum and then stored at −20˚C for further analysis.

2.9. Microbial Analyses

2.9.1. Sterilization and Aseptic Techniques

All glasswares were washed thoroughly with detergent, rinsed and sterilized at 121˚C at 15 psi for 15 minutes. The working surface was disinfected with 70% (v/v) alcohol. All media were prepared according to manufacturer instruction, sterilized at 121˚C at 15 psi for 15 minutes. The mouth of each flask was flame sterilized before pouring the media into the sterile petri dishes.

2.9.2. Preparation of Inoculum

About 1 g of each sample was diluted up to ten folds in sterile test tube containing 9 ml of 0.85% saline solution (NaCl). About 100 micrograms of 2 different dilution factors 105 and 106 were pipetted into the plate.

2.9.3. Preparation of Different Culture Media

For the preparation of the different culture media, the different constituents of the medium were weighed, put in a glass jar and then poured in distilled water according to the volume prepared. Then the mixture was then autoclaved at 121˚C, 15 psi for 15 and then cooled before pouring into each petri dish. Each medium was prepared in 500 ml of water.

Preparation of media for *Escherichia coli*

About 18.5 g of Eosin Methylene Blue was measured into a glass jar and 500 ml of distilled water was poured into it. The mixture was autoclaved at 15 psi for 15 min. The mixture was left to cool before pouring into each petri dish.

Preparation of media for Bacteroides media

Brain heart infusion agar media (BHI) was supplemented for the growth of Bacteroides. About 18.5 g of BHI, 2.5 g of yeast extract and 500 ml of water were measured into a glass jar. The mixture was autoclaved at 121˚C, 15 psi for 15 min. The mixture was cooled and 0.25 ml of blood, 0.5 mg of menadione, 20 ml of vancomycin and 0.2 mg of gentamicin were then added.

Preparation of media for bifidobacterial

Man Regosa Sharpe (MRS) agar media was to culture Bifidobacterial. The media was supplemented with L cysteine, tween 80 and cyclohexamide. About

26 g of MRS, 0.5 ml of tween 80 and 500 ml of water were measured into a glass jar and the mixture was autoclaved at 121˚C, 15 psi for 15 min. When the mixture was cooled, 25 mg of Lcysteine, and 2 ml of cyclohexamide were added.

Preparation media for lactobacillus

Man Regosa Sharpe (MRS) agar media supplemented with tween 80 and cyclohexamide was used in culturing Lactobacillus. About 26 g of MRS agar, 0.5 ml of tween 80 and 500 ml of distilled water were measured into a glass jar. The mixture was then autoclaved 121˚C, 15 psi for 15 min. When the mixture was cooled, 2 ml of cyclohexamide was added to the mixture.

Total bacterial count

Plate count agar was used to quantify the total bacteria in the fecal sample. About 10 g of plate count agar and 500 ml of distilled water were measured into a glass jar. The mixture was autoclaved at 121˚C, 15 psi for 15 min. The mixture was cooled before it was poured into a petri dish.

2.9.4. Sowing

1) Surface Seeding

Seeding was done in one layer and consisted of placing 1 ml of sample in a Petri dish before introducing the culture medium. This type of seeding is ideal for strains such as *Escherichia coli.*

2) Immersion

Here the seeding was done with a double layer. This type of seeding is suitable for anaerobes; a quantity of culture medium was introduced into the petri dish and then left to solidify, then 1 ml of sample was introduced. Finally, the culture medium was further added and left to solidify. This type of seeding is ideal for the growth of Bacteroides, Bifidobacterial, and Lactobacilli. All the petri dishes were later sealed to prevent oxygen since these bacteria are anaerobes. All petri dishes were incubated 37˚C for 24 for the EMB, Plate Count agar, BHI media, MRS and MRS+ cysteine agar.

2.9.5. Plate Count

After bacterial growth was seen on the surface of the media, the CFU (colony forming unit) was obtained by counting the number of bacterial colonies found on the media and inserting the values into the formula below [15].

CFU = number of bacterial colonies × dilution factor volume of inoculum used

2.9.6. Characterization of the Isolates

1) Catalase test

Catalase is an enzyme produced by most micro-organism that breaks down hydrogen peroxide into water and oxygen which produces gas bubbles [16].

$$2H_2O_2 \rightarrow 2H_2O_2 + O_2$$

The test was done by placing a drop of 3% hydrogen peroxide solution onto a slide. Using a sterilized needle, a colony was randomly chosen from a media and placed into the drop. The isolate which did not give gas bubbles was characterized as

catalase negative, those that produced bubbles were characterized as positive [16].

2) Gram staining

Gram staining is important in identifying and classifying bacteria depending on their characteristic. The principle is based on the ability of the cell walls to retain stain. Isolates were collected from the colony that was chosen for catalase test. The bacteria were fixed onto a slide by placing a loop full of water onto the slide. The loop was sterilized by placing the loop on to the flame until it bright hot then allowed to cool down. Using this loop, the colony that was chosen for the catalase test was scraped and smeared onto the slide. They were left to air dry and the bacteria were fixed by passing it over a flamed 3 times. Crystal violet (primary slide) was applied onto the slide for 30 to 60 s to color the slide purple. The slides were then washed away with running water to stop the reaction. Lugol iodine (mordant) was then applied for 30 to 45 s and washed off using running water 70% alcohol was used to decolorize the bacteria by removing the excess stain. The slides were then washed with running water to stop the reaction. Diluted safranin was added to the slide for 10 to 15 s and then washed off with running water. The slides were then air-dried. The prepared slides were then observed under the light microscope with the magnification of 100× using immersion oil. Bacteria which has pink color were gram-negative and those of purple color were gram-positive.

2.10. Biochemical Analysis of Serum

2.10.1. Estimation of Lipid Profile

The triglyceride and total Cholesterol levels were determined by the Buccolo colorimetric method using Chrono lab test kit [17]. The very low density (VLDL) and low density (LDL) lipoproteins from serum or plasma were precipitated by phosphotungstate in the presence of magnesium ions. After centrifugation, the supernatant containing high density lipoproteins (HDL) was determined using the total cholesterol enzymatic reagent [18]. LDL-cholesterol was calculated from measured values of total cholesterol, triglycerides and HDLcholesterol according to the relationship: [LDL-chol] = [total chol] − [HDL-chol] − [TG]/5 and [TG]/5 was an estimate of VLDL-cholesterol.

2.10.2. Estimation of Serum Enzymes

The activity of (ALP) was determined by the Reitman and Frankel colorimetric [19] UV Kinematic determination of alkaline phosphatase in serum based upon IFCC recommendation using INMESCO test kit. Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) were measured using the chrono lab enzymatic kit [20]. The concentration of creatinine was estimated using chrono lab enzymatic kit [21].

2.11. Statistical Analysis

Analysis was done in duplicates. The various data collected was imported into Microsoft Excel where the mean and standard deviation were calculated. The

data was then subjected to one-way analysis of variance (ANOVA), using GraphPad InStat version 3.10 in order to evaluate the statistical significance of the mean. Student-Newman-Keuls tests were used to calculate the significant difference. The result of this test was presented in tables and figures. A probability value at $p < 0.05$ was considered statistical significance.

3. Results

3.1. Food Intake

The weekly food intake for rats fed with restricted and normal diets is represented in Table 2. Generally, a significant ($p < 0.05$) increase in food intake was recorded with all groups during the four weeks except for carbohydrate and fat restricted diet and carbohydrate and protein restricted diet which food intake increased right up to the third week before significantly decreasing the fourth week.

Table 2. Weekly food intake of feed in grams.

Groups	Week1	Week2	Week3	Week4
ND	34.76 ± 3.92^{b}	48.00 ± 9.33^{cd}	50.00 ± 10.00^{b}	60.86 ± 6.87^{bc}
FRD	41.70 ± 10.42^{b}	$41.71 \pm 7.93^{c,d}$	50.57 ± 7.73^{b}	53.14 ± 15.13^{b}
PRD	34.34 ± 7.78^{b}	$26.86 \pm 9.20^{a,b}$	47.43 ± 7.97^{b}	53.71 ± 8.15^{b}
CRD	43.40 ± 9.90^{b}	52.00 ± 11.63^{d}	46.57 ± 6.77^{b}	66.29 ± 11.65^{bc}
PFRD	38.22 ± 13.44^{b}	36.00 ± 10.26^{abcd}	46.57 ± 8.55^{b}	70.00 ± 9.05^{bc}
CFRD	51.20 ± 2.12^{b}	55.43 ± 8.32^{d}	65.29 ± 2.87^{c}	76.00 ± 7.72^{c}
CPRD	13.12 ± 8.31^{a}	22.86 ± 1.99^{a}	24.86 ± 4.92^{a}	18.86 ± 3.55^{a}

N = 4, all values assigned the same letter are not significantly different from each order at $p < 0.05$ while those assigned different letters are significantly different at $p < 0.05$, ND: Normal diet, FRD: Fat restricted diet, CRD: Carbohydrate restricted diet, PRD: protein-restricted diet PFRD: Protein and fat restricted diet, CFRD: Carbohydrate and fat restricted diet.

The food intake among groups showed that carbohydrate and protein restricted diet (CPRD) presented the lowest ($p < 0.05$) values on week one. In the second week protein restricted diet (PRD), and carbohydrate and protein restricted (CPRD) showed the lowest ($p < 0.05$). In the third week carbohydrate and fat restricted diet (CFRD) reported a significantly higher food intake compared to the other groups while carbohydrate and protein restricted diet (CPRD) presented the lowest value. In the last week carbohydrate and protein restricted diet (CPRD) was the group with the lowest food intake compared to the other ones.

3.2. Weekly Weights

The average body weights (weekly basis) of the different groups of rats are presented in Figure 1. A significant ($p < 0.05$) decrease in body weight was regis-

tered in all groups, except for the control group where this parameter was significantly increased.

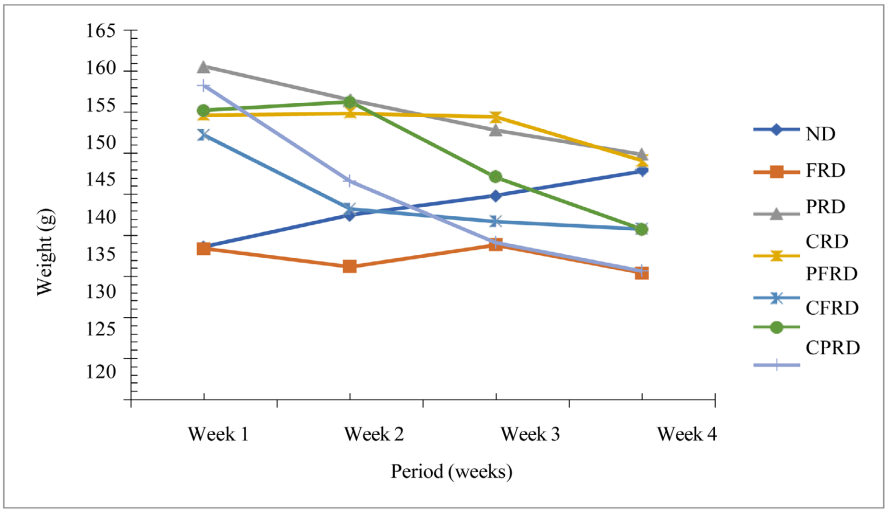
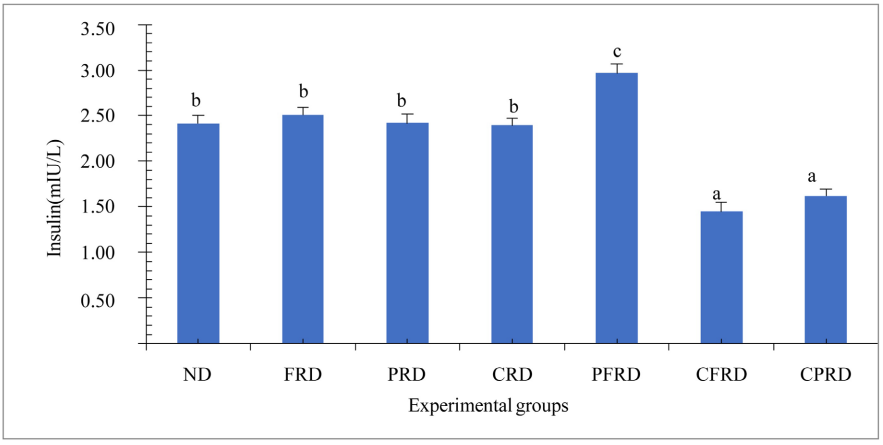


Figure 1. The effect of macronutrient restriction on weight of rats.

3.3. Insulin Levels

The insulin level is presented in **Figure 2**. About 50% of the test groups had insulin levels that were either high or low compared to the control group. Insulin levels were found to be significantly ($p < 0.05$) lower in the carbohydrate and fat restricted (CFRD), and carbohydrate and protein restricted (CPFD) groups while a significant ($p < 0.05$) increase was recorded in protein and fat restricted groups compared to the normal.



n = 2, Values are expressed as mean ± standard deviation. The values assigned the same super-script are significantly not different ($p \leq 0.05$) from each other and those assigned different superscripts are significantly different; ND Normal diet, FRD: Fat restricted diet, CRD: Carbohydrate restricted diet PFRD: Protein and fat restricted diet, CFRD: Carbohydrate and fat restricted diet, CPRD: Carbohydrate and protein restricted diet. CFU: Colony forming unit.

Figure 2. Insulin levels for rats fed with macronutrient restricted diet.

3.4. Oral Glucose Tolerance Test

The oral glucose test is presented in Figure 3. Generally, the glucose levels of all groups increased after admission of glucose (2 g/kg body weight) except for the control group and decreased as time progressed. A significant ($p < 0.05$) difference in the oral glucose tolerance was found among the groups. Protein restricted diet (PFRD) recorded a significantly ($p < 0.05$) higher fasting glucose level than the rest of the group. After glucose administration the carbohydrate restricted diet record a significantly ($p < 0.05$) higher glucose level.

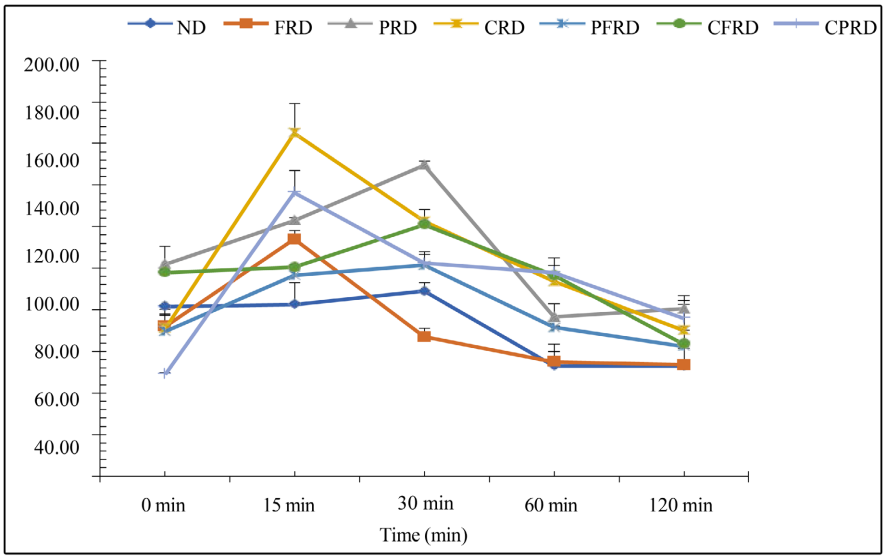


Figure 3. Oral glucose concentration of rats fed with restricted die.

3.5. Microbial Analysis

3.5.1. Cultural Characteristics of the Bacteria Isolates

Table 3 shows the morphological characteristics of isolates such as margin, elevation, shape and color. All colonies were round, entire and flat except for the lactobacillus colonies that were convex. Colonies that grew in the Brain Heart Infusion Agar (BHI) and the Man Regosa Sharpe (MRS) + Cysteine agar were red in color and those in the Eosin methylene blue (EMB) and Man Regosa Sharpe (MRS) were black and white in color respectively.

3.5.2. Biochemical Characteristics of the Isolates

The biochemical characteristics of the isolates such as catalase test and gram stain are described in Table 4. The catalase test reveals that all colonies formed on the culture media were catalase negative except for those formed on brain heart infusion agar (BHI). Colonies formed on Man Regosa Sharpe (MRS) and Man Regosa Sharpe (MRS) + cysteine agar were gram positive while those formed on Eosin methylene blue (EMB) and Brain Heart Infusion (BHI) were gram negative. When viewed under the microscope all isolates appeared to be rod shaped except isolates from MRS + cysteine which where branched rods.

Table 3. Colony morphology.

Culture medium	Morphology isolates	ND	FRD	PRD	CRD	PFRD	CFRD	CPRD
BHI	Color	Red	Red	Red	Red	Red	Red	Red
	Elevation	Flat	Flat	Flat	Flat	Flat	Flat	Flat
	Shape	Round	Round	Round	Round	Round	Round	Round
	Margin	Entire	Entire	Entire	Entire	Entire	Entire	Entire
MRS agar + cysteine	Color	Red	Red	Red	Red	Red	Red	Red
	Elevation	Flat	Flat	Flat	Flat	Flat	Flat	Flat
	Shape	Punctiform	Punctiform	Punctiform	Punctiform	Punctiform	Punctiform	Punctiform
	Margin	Entire	Entire	Entire	Entire	Entire	Entire	Entire
MRS agar	Color	White	White	White	White	White	White	White
	Elevation	Convex	Convex	Convex	Convex	Convex	Convex	Convex
	Shape	Round	Round	Round	Round	Round	Round	Round
	Margin	Entire	Entire	Entire	Entire	Entire	Entire	Entire
EMB agar	Color	Black	Black	Black	Black	Black	Black	Black
	Elevation	Flat	Flat	Flat	Flat	Flat	Flat	Flat
	Shape	Circular	Circular	Circular	Circular	Circular	Circular	Circular
	Margin	Entire	Entire	Entire	Entire	Entire	Entire	Entire

ND: Normal diet, FRD: Fat restricted diet, CRD: Carbohydrate restricted diet PRD: protein restricted diet PFRD: Protein and fat restricted diet, CFRD: Carbohydrate and fat restricted diet, CPRD: Carbohydrate and protein restricted diet.

Table 4. Biochemical characteristics of isolated gut microbiota.

Culture Medium	Catalase	Gram Stain	Shape	Culture Medium
Brain heart infusion agar (supplemented)	+	–	Rods	Brain heart infusion agar (supplemented)
MRS+ cysteine	–	+	Branched rods	MRS+ cysteine
MRS	–	+	Rods	MRS
EMB	–	–	Rods	EMB

EMB: Eosin Methylene Blue, MRS De Man, Rogosa and Sharpe.

3.5.3. Microbial Composition

The microbial analysis of rat fed with macronutrient restricted diet and a normal diet is presented in Table 5. The composition of Bacteroides was significantly ($p < 0.05$) decreased in the fat restricted (FRD) and protein restricted (PRD) groups. Meanwhile this parameter was significantly ($p < 0.05$) increased in the carbohydrate restricted (CRD) and carbohydrate and protein restricted (CPRD) groups

compared to the control group. For Lactobacillus, its composition was increased ($p < 0.05$) in protein and fat restricted (PFRD) groups, and significantly ($p < 0.05$) decreased in the carbohydrate restricted group (CRD) compared to the control group. A significant ($p < 0.05$) increase in Bifidobacterial composition was recorded in the protein restricted (PRD) and protein and fat restricted (PFRD) groups compared to the normal. However, this parameter significantly ($p < 0.05$) decreased in the carbohydrate restricted CRD, carbohydrate and fat restricted (CFRD), and protein and fat restricted diet compared to the control group. Carbohydrate restricted (CRD) and carbohydrate and fat restricted (CFRD) groups recorded a significant ($p < 0.05$) increase in *E. coli* composition compared to the normal. The total bacterial count was significantly ($p < 0.05$) low in the carbohydrate restricted (CRD), carbohydrate and fat restricted (CFRD) and carbohydrate and protein restricted (CPRD) groups compared to the normal.

Table 5. Results for microbial analysis of bacteria isolates.

Groups	Bacteroides	Lactobacillus	Bifidobacterium	*E. coli*	TBC
	(CFU)	(CFU)	(CFU)	(CFU)	(CFU)
ND	40.50 ± 6.36^{bc}	73.00 ± 5.65^{b}	59.50 ± 7.77^{b}	30.5 ± 2.12^{a}	129.50 ± 3.53^{c}
FRD	14.00 ± 5.65^{a}	60.00 ± 1.41^{b}	63.50 ± 3.53^{b}	26.5 ± 7.77^{a}	139.50 ± 12.02^{c}
PRD	19.00 ± 5.65^{a}	71.50 ± 9.19^{b}	92.00 ± 7.07^{c}	29 ± 1.41^{a}	148.00 ± 11.31^{c}
CRD	52.50 ± 2.12^{c}	33.50 ± 6.36^{a}	47.00 ± 2.82^{a}	51.5 ± 3.53^{b}	48.00 ± 11.31^{ab}
PFRD	39.50 ± 7.77^{bc}	102.50 ± 4.94^{c}	100.00 ± 2.82^{c}	39.5 ± 0.70^{a}	72.00 ± 11.31^{b}
CFRD	22.50 ± 6.36^{ab}	78.00 ± 4.24^{b}	$41.5.00 \pm 3.53^{a}$	82.00 ± 8.48^{c}	34.50 ± 6.36^{a},
CPRD	69.50 ± 9.19^{d}	67.50 ± 3.53^{b}	38.00 ± 2.82^{a}	26.00 ± 2.82^{a}	67.00 ± 7.07^{ab}

n = 2, Values are expressed as mean ± standard deviation. The values assigned the same superscript are significantly not different ($p \leq 0.05$) from each other and those assigned different superscripts are significantly different; ND Normal diet, FRD: Fat restricted diet, CRD: Carbohydrate restricted diet PFRD: Protein and fat restricted diet, CFRD: Carbohydrate and fat restricted diet, CPRD: Carbohydrate and protein restricted diet. CFU: colony forming unit.

3.6. Lipid Profile

The effect of restricting macronutrient on serum lipid profile is presented in Table 6. A significant ($p < 0.05$) increase in high density lipoprotein (HDL) was recorded in the rats fed with protein and fat restricted diet meanwhile carbohydrate and protein restricted (CPRD), carbohydrate and fat restricted (CFRD), fat restricted diet (FRD) and carbohydrate restricted diet (CRD) where significantly ($p < 0.05$) decreased. With Very low density lipoprotein (VLDL), carbohydrate and fat restricted diet (CFRD) was significantly decreased. Concerning low density lipoprotein (LDL) a significant ($p < 0.05$) increase was recorded in the protein restricted group compared to the control group. For cholesterol, a significant ($p < 0.05$) decrease was recorded in rats fed with carbohydrate restricted diet (CRD) compared to the normal. Protein restricted diet (PRD), carbohydrate

restricted diet (CRD) and carbohydrate and fat restricted diet (CFRD) recoded significantly ($p < 0.05$) low values for triglycerides compared to the control while carbohydrate and protein restricted diet (CPRD) and protein and fat restricted diet (PFRD) values where significantly ($p < 0.05$) higher.

Table 6. The effect of macronutrient restriction on lipid profile.

Groups	HDL (U/L)	VLDL (U/L)	LDL (U/L)	Cholesterol (U/L)	TG (U/L)
ND	68.2 ± 7.5^{d}	22.77 ± 5.88^{bc}	30.95 ± 4.24^{a}	121.44 ± 3.98^{bcd}	111.67 ± 4.41^{c}
FRD	40.4 ± 2.0^{b}	23.13 ± 6.24^{bc}	44.50 ± 8.56^{ab}	108.83 ± 8.48^{abc}	119.44 ± 3.54^{c}
PRD	64.5 ± 1.4^{de}	19.93 ± 4.42^{bc}	49.97 ± 9.48^{b}	134.41 ± 7.57^{cd}	99.67 ± 12.02^{b}
CRD	30.8 ± 4.7^{a}	15.67 ± 1.22^{b}	42.14 ± 7.65^{ab}	88.65 ± 11.11^{a}	78.33 ± 9.43^{a}
PFRD	74.7 ± 1.9^{e}	29.33 ± 4.19^{c}	41.00 ± 6.91^{ab}	145.05 ± 9.59^{d}	146.67 ± 12.02^{d}
CFRD	41.3 ± 3.5^{b}	8.33 ± 0.19^{a}	40.93 ± 5.89^{ab}	97.39 ± 8.96^{ab}	75.56 ± 0.96^{a}
CPRD	53.5 ± 7.8^{c}	31.00 ± 7.80^{c}	41.98 ± 8.18^{ab}	128.56 ± 14.31^{bcd}	165.33 ± 2.36^{d}

n = 4, The values are expressed as the mean ± standard deviation of the mean. The values assigned the same letter are not significantly different from each other while those assigned different letters are significantly different ($p < 0.05$) ND: Normal diet, FRD: Fat restricted diet, CRD: Carbohydrate restricted diet PFRD: Protein and fat restricted diet, CFRD: Carbohydrate and fat restricted diet, CPRD: Carbohydrate and protein restricted diet, HDL: high density lipoprotein, VLDL: very low density lipoprotein, LDL: low density lipoprotein, TG: triglyceride.

3.7. The Serum Enzymes and Other Biochemical Parameter

The effect of macronutrient restriction on serum enzyme is presented in Table 7. No significant difference was recorded between the creatinine value of the rats fed with nutrient restricted diet compared to the control group (ND). There was a general increase recorded in AST levels among all groups.

Table 7. The effect of macronutrient restriction on serum enzymes and other biochemical parameter.

Diets	Creatinine (mg/dl)	AST (μ/L)	ALT (μ/L)	ALP (μ/L)
ND	$3.7 \pm 1.3^{a,b}$	66.62 ± 4.92^{b}	10.50 ± 4.12^{a}	116.96 ± 10.41^{bc}
FRD	$4.4 \pm 1.6^{a,b}$	64.74 ± 4.91^{b}	15.60 ± 5.20^{a}	150.63 ± 21.49^{e}
PRD	$3.7 \pm 1.5^{a,b}$	69.43 ± 4.94^{b}	14.29 ± 5.00^{a}	103.65 ± 28.01^{a}
CRD	3.1 ± 0.7^{a}	59.41 ± 6.11^{a}	19.69 ± 2.90^{a}	152.48 ± 20.84^{e}
PFRD	6.8 ± 3.3^{b}	85.81 ± 3.31^{c}	35.00 ± 5.89^{b}	121.38 ± 37.78^{c}
CFRD	2.9 ± 0.6^{a}	70.30 ± 6.23^{c}	14.72 ± 1.29^{a}	112.86 ± 8.46^{b}
CPRD	3.3 ± 1.0^{a}	95.43 ± 6.24^{d}	16.91 ± 2.38^{a}	134.05 ± 20.19^{d}

n = 4, the values are expressed as the mean ± standard deviation of the mean. The values assigned the same letter are significantly the same while those assigned different letters are significantly different ($p < 0.05$) ND: Normal diet, FRD: Fat restricted diet, CRD: Carbohydrate restricted diet PFRD: Protein and fat restricted diet, CFRD: Carbohydrate and fat restricted diet, CPRD: Carbohydrate and protein restricted diet, AST: Aspartate transaminase, ALT: Alanine transaminase, ALP: Alkaline phosphatase.

4. Discussion

4.1. Food Intake and Body Weight

The body needs macronutrients to provide energy, build and repair tissues, insulate organs and make cell membranes. In this study, ad libitum consumption of macronutrient restricted diet for 4 weeks produces a decrease in body weight compared to the control group despite an increase in food intake. Among all the groups that experienced weight loss, CPRD lost 15% of its weight during the experiment followed by CFRD group (10% of their weight). These two groups also had the highest (CFRD) and the lowest (CPRD) feed consumption. The weight loss experienced in the CFRD group is close to the low carbohydrate/high fat diet of Noakes *et al.* [22] and Ludwig *et al.* [23]. The weight loss in this group can be attributed to its high fat content. High fat diet is extremely satiating, help curb hunger and craving hence helps prevent overeating [22]. This also explains the low feed consumption of this group. The weight loss experienced by the CFRD groups mimics the high protein diet group of Westerter *et al.* [24]. This weight loss can be attributed to the fact that, high protein diet increases energy expenditure by increasing diet induced thermogenesis, which can lead to negative energy balance—a state of greater energy output than input [25]. The high food intake of this group could be the effect of high ghrelin experienced in high protein diet. Ghrelin is a hormone that increases appetite and Leidy *et al.* [26] found out that these hormone increase with high protein intake leading to a great frequency of food consumption. This also explains the high feed intake in this group.

4.2. Insulin Levels and Glucose Tolerance Test

The role of insulin in the blood is to regulate blood sugar level. The insulin level in this study was found to be higher in the PFRD group and lower in the CRFD and CPRD groups. The increase in the fasting insulin levels experienced in the PFRD group was inconsistent with that of Solon-Biet *et al.* [27]. They found that fasting insulin levels in mice decreased with increasing dietary carbohydrate content after 8 weeks on experimental diets [27]. Also, Axen and Axen [28] experienced higher fasting insulin levels when comparing a 6% carbohydrate diet with a chow diet that had 70% carbohydrate after 6 - 7 weeks of exposure. Similarly, the fasting insulin levels of rats were not strongly linked to dietary carbohydrate content between 5% and 60% carbohydrate intake [29]. The reason why the insulin level was high in this present study can be attributed to the high carbohydrate content of PFRD. The body strives to regulate the glucose produced during the break down of carbohydrate by releasing insulin.

Low levels of insulin found in the CPRD and CFRD are consistent with a study by Anne *et al.* that shows a decreased production of glucose in fasting state after both polyunsaturated fatty acid (PUFA) and saturated fatty acid (SFA) are ingested in high-fat diet [30]. An observational study by Linn *et al.* showed reduced insulin sensitivity, greater endogenous glucose production and greater net

gluconeogenesis in high protein diets [31].

4.3. Microbial Analysis

One major function of gut microbiota is the breakdown of macronutrient to release metabolites that are useful to both the host and the microorganism itself. Bacteroides, Bifidobacterial, lactobacillus and *E. coli* are examples of these bacterial found in the gut. This microorganism show distinctive characteristic when cultured in the laboratory. In this study supplemented BHI agar was used to cultivate Bacteroides. Colonies formed where red, round flat and entire, which were negative on catalase test and were pink rods when viewed under the microscope. This is consistent with other studies that revealed that Bacteroides are gram negative rod [32], and catalase negative [33].

Both Bifidobacterial and Lactobacillus can be cultivated with MRS. These organisms can be differentiated when cysteine is added to the medium. This promotes the growth of Bifidobacteria that forms red punctiform colony, that is catalase negative and gram positive branched rods while lactobacillus forms white colonies that are round and convex, that are catalase negative and gram positive rods. Similar results were obtained in this study and also on infant feces by Biavati *et al.* [34]. EMB agar used in this study produced black, flat and round colonies which are catalase negative and gram-negative rods. The identification of E.Coli was consistent with those of Holt-Harris [35].

4.4. Microbial Composition

Diet is arguably one of the most important forces shaping the gut microbiota. Of all the factors influencing gut microbiota composition and function throughout all life stages, diet is key in modulating abundances of specific bacterial species and their functions [36]. The composition of some gut bacteria in this study changed when some macronutrients were restricted. The composition of Lactobacillus and Bifidobacterial increased when the proportion of carbohydrate in the diet was high and decreased when it was low. Matsumoto *et al.* [37], Priebe *et al.* [38] and Zhong *et al.* [39] reported similar results with humans fed with probiotic milk. Carvalho—Wells also reported an increase in intestinal Bifidobacteria and lactic acid bacteria when maize-based whole grain breakfast cereal is consumed [40]. The increase could be explained by the fact that, these organisms are saccharolytic bacteria that degrade carbohydrates as reported by Nordmann *et al.* [41]. Protein is an important source of essential nutrients and is necessary to maintain a normal body and health [42]. However, chronic consumption of protein can result in serious pathological and degenerative diseases involving gut microbiota [43]. In this study, the relative abundance of Bacteroides decreased in both PRD and CFRD groups. In addition to the decrease in the Bacteroide composition an increase in bifidobacterial and *E. coli* was seen in PRD and CFRD respectively. The decreased abundance of Bacteroides in the PRD group is in line with those of Mu *et al.* [44]. The CFRD in this study mimics the high

protein diet group of Leidy *et al.* [26] and Mu *et al.* [44]. The increase in *E. coli* can be attributed to the fact that, high protein diet induces a reduction of both propionateand butyrate-producing bacteria and thus the production of propionate and butyrate [45]-[47]. These metabolites play a key role in inhibiting the growth of pathogens in the gut. the reduction of these bacteria in a high protein diet creates a favorable environment for the growth of pathogenic bacteria such as Escherichia, Enterococcus, and Streptococcus.

This study reveals a decrease in Bacteroides in FRD group but this microorganism increased in the CPRD group. The increase in Bacteroides in the CPRD group is consistent with the high-fat diet of Wu *et al.* [48] and Drasar *et al.* [49]. Both studies suggested that a high-fat diet increases total anaerobic microflora and counts of Bacteroides. In addition to the decrease in Bacteroide in the CPRD group, an increase in Bifidobacterial was also recorded. This confirms the results of Murphy *et al.* [50] and Lecomte *et al.* [51]. Lecomte *et al.* found out that a high-fat diet resulted in propionate and acetate producing bacteria (Clostridial, Bacteroides, and Enterobacterial) [26]. Murphy *et al.* [50] study in mice revealed a decrease in the bifidobacterial population. The result of this study could be explained by the fact that when dietary fat content is increased, there is usually a low content of other dietary compounds such as carbohydrates and fiber. Low carbohydrate and fiber diets could reduce energy substrates for beneficial bacteria growth such as bifidobacterial.

4.5. Micronutrient Restriction and Biochemical Parameters

In this study, macronutrient restriction had a great impact on HDL and triglyceride levels. Compared to the normal diet, HDL levels decreased with FRD, CRD, CFRD, and CPRD but increased with PFRD. A triglyceride reduction was recorded with PRD, CRD, and CFRD but increased with PFRD. Meanwhile, CRD and CFRD decreased cholesterol and very low-density lipoprotein respectably there was an increase in LDL levels but these increases were not significant except for PRD.

The decrease in HDL and total cholesterol reported in this study with CRD is consistent with those observed by Hu *et al.* [52] and Bazzano *et al.* [53]. A major concern that has been frequently raised about low-carbohydrate diets is their potential to elevate LDL cholesterol levels [54]. This study found an increase in LDL cholesterol levels among rats fed with low carbohydrate diet. However, this increase was not significant to the normal. Indeed, a high intake of total carbohydrate is associated with higher triglyceride concentrations in adults [55] and children [56]. In this study, the increase in triglyceride level was recorded in the PFRD. This finding is consistence with those of Parks *et al.* [57]. This increase can be attributed to an increase in triglycerides levels caused by increased dietary carbohydrates, particularly simple sugars and starches with high glycemic index [58].

The role of fat intake in serum lipid levels differs according to the type of fat

consumed. Indeed, the fatty acid profile of the diet seems to be the major determinant of serum cholesterol concentrations. HDL levels decreased with FRD in this study. Alice *et al.* also found a negative association between low fat intake and HDL humans [59]. This decrease in HDL concentrations, presumably resulted in a decrease in fat intake [60]. High-fat (CPRD) for 28 days resulted in decreased levels of HDL and increased triglyceride in the rats. This result is consistent with those of Kalaivanisailaja *et al.* [60]. The increase in plasma triglyceride can be due to a decrease in the activity of lipoprotein lipase [60].

Focusing on proteins, the PRD group recorded an increase in LDL. This finding is in contrast to those recorded by Bruna *et al.* [61]. Fontes *et al.* observed a greater decrease in serum levels of total cholesterol and LDL-c in chronic kidney disease patients receiving low protein diet. A decrease in LDL was expected in this since a decreased intake of animal protein helps decrease serum cholesterol levels. The high protein group in this study recorded a decrease in HDL, VLDL, total cholesterol and triglyceride level. This finding is coherent with those of Lunacastilo *et al.* [62]. These decreases can be attributed to reduction in chylomicron formation due to decreased fat intake, faster chylomicron clearance by increasing LPL stimulation, effectiveness of lipid oxidation in the liver, and decreased lipid synthesis [63].

4.6. Serum Enzymes and Macronutrient Restriction

A remarkable increase in ALT in the PFRD was recorded in this study. This finding is consistent with those of Purlins *et al.* [64]. This could be due to the presence of high carbohydrate in this diet. An influx in carbohydrate directly affects ALT levels as this enzyme is involved in pyruvate metabolism [64]. The highest level of AST was recorded by CPRD in this study. This result is in accordance with the findings of Welch *et al.* [65] who showed an increased AST level with high fat diet in euthyroid and thyroid-altered rats. This increase can be due to dehydration and high protein content. This enzyme plays a key role in the metabolism of amino acids in all species [66]. The highest ALP value was recorded by the CRD group. This finding is in accordance with Zhou *et al.* [67] who demonstrated that a diet high in fat causes an increase in ALP levels in rats suffering from non-alcoholic fatty liver disease.

5. Conclusion

In conclusion, the present study showed the associations between macronutrient intake, gut microbiota, and serum lipid and enzyme profile in Wistar rats. The research thus far reveals that gut Microbiome, serum lipid and enzyme profile can respond to an altered diet, potentially facilitating the diversity of human dietary lifestyles. Gut microbial dysbiosis was found among all the groups as a shift in the microbiota composition was recorded. Indeed, macronutrient restriction affects lipid profile. HDL and triglyceride were highly affected by nutrient restriction. Groups that received diets high in macronutrient ratio had a greater

effect on lipid profile than those on low macronutrient ratio. Serum enzymes were also affected by nutrient restriction. ALP was one enzyme found to be greatly affected by macronutrient restriction. Overall, we can conclude that current evidence for developing optimal dietary interventions targeting diet control via the gut microbiota is still in its infancy and does not capture the complexity of the integration of a whole-diet approach, the microbial and the host's metabolic phenotype. Implementation of targeted, precision nutrition intervention strategies or dietary guidelines for individuals or subgroups in public health is still more remote and will require insight into the mechanisms involved in (non-) response to dietary intervention, implying that we need to go beyond prediction models. Detailed individual phenotyping and gaining insights into the balance between carbohydrate and protein fermentation by the gut microbiota as well as the site of fermentation in the colon are the key. The baseline gut microbial profile may be a predictor of an individual's response to dietary interventions. Future research in this field should consider the detailed characterisation of both microbial and metabolic phenotypes as well as their interaction. Gut microbiome-associated effects on host metabolism may be related to fermentation products of carbohydrates and proteins. Understanding how to optimally balance proteolytic and saccharolytic fermentation and gaining insight into the importance of the site of colonic fermentation will give insight into the interplay between diet, gut microbiome and metabolic processes. Understanding the mechanisms of the differential responses to diet is essential to move forward in the field of precision nutrition. Although both the amount and quality of knowledge have evolved rapidly in recent years, we still only see the tip of the iceberg. Dietary macronutrients and the gut microbiome would be a precision nutrition approach to improve health.

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Conflicts of Interest

The authors declare no conflict of interest.

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