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Investigating of Moringa Oleifera Role on Gut Microbiota Composition and Inflammation Associated with Obesity Following High Fat Diet Feeding

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Abstract

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AIM: The alteration in the gut microbial community has been regarded as one of the main factors related to obesity and metabolic disorders. To date, little is known about Moringa oleifera as a nutritional intervention to modulate the microbiota imbalance associated with obesity. Therefore we aim to explore the role of aqueous Moringa oleiferous leaf extract on Lactobacilli and Bifidobacteria in high-fat diet-induced obesity and to investigate whether any restoration in the number of caecal Lactobacilli and Bifidobacteria could modulate obesity-induced inflammation.

METHODS: Young Swiss albino mice were divided into three groups according to their diet. Two of them were fed on either high fat diet or high fat diet+aqueous extract of Moringa oleifera leaf, while the third group was fed on the control diet. Bacterial DNAs were isolated from the mice digesta samples for bacteria level estimation using Quantitative real-time polymerase chain reaction along with serum interleukin-6 and lipid profile

RESULTS: Compared to the normal control mice, high-fat diet feeding mice showed significantly reduced intestinal levels of Bifidobacteria, and increased body weight, interleukin 6, and levels of Lactobacilli. Upon treatment with Moringa oleifera, body weight, interleukin 6, and both bacteria levels were significantly restored

CONCLUSIONS: Our findings suggest that Moringa oleifera aqueous leaf extract may contribute towards the pathophysiological regulation of weight gain, inflammation associated with high-fat-induced-obesity through gut bacteria modulation.

Introduction

Obesity is well known to be a consequence of contributing factors, including hereditary, metabolic, behavioural and environmental factors [1] [2] [3]. Gut microbiota has been viewed as one of the main environmental factors identified with obesity and metabolic disorders [4] [5] [6]. Indeed the major cause of the development of obesity is the imbalance between energy intake and energy expenditure. The microorganisms residing in the gut influence the entire body metabolism by affecting such energy balance, in addition to other mechanisms including inflammation, gut barrier function, integrate peripheral and central nutritionally relevant information, therefore, increase adiposity and body weight [4] [7] [8] [9].

Snaz et al., and other researchers have supported this view and gained great insight into the relationship between gut bacteria and metabolic disorders [10]. However, certain phyla and classes of bacteria are associated with the improved transfer of calories from the diet to the host, and with alterations in the host metabolism of absorbed calories [6] [11] [12] [13] [14]. They ferment dietary polysaccharides and convert them into monosaccharides and short-chain fatty acids (SCFAs). These metabolites are subsequently absorbed and react as a source of energy by the host. Moreover, SCFAs, through free fatty acid receptors 2 (FFAR2) and 3 (FFAR3), are thought to participate in the regulating of different gut hormones. In addition to their vital role in nutrient absorption and metabolism regulation they control the proliferation of some pathogenic bacteria existed in the intestinal tract [15], induce the immune system, contribute in the production of vitamins and enzymes

such as vitamin K and biotin, and in the synthesis of useful compounds for the mucosa and cell renewal [16] [17].

Lactobacillus (Lb) and Bifidobacteria are the major component of gut microbiota that are recognised for their basic roles in normal physiological processes. Moreover, they include such probiotics as Lactic acid bacteria (LAB), which have been proved benefit effects to human health. Interestingly, they show an alteration in diet-induced obesity via different mechanisms. Those beneficial intestinal Lactobacilli and Bifidobacteria can synthesise bioactive isomers of conjugated linoleic acid that have antidiabetic, anti-atherosclerotic, immunomodulatory, and anti-obesity properties [18]. For the close connection between gut microbiota composition and metabolic disorders, more studies are needed to focus on how the composition of gut bacteria community be modified to reduce the risk of obesity and the associated metabolic changes.

Currently, considerable efforts have been made using plants as traditional natural medicines for reducing body weight and healing many diseases. Among them is *M. oleifera* that belongs to the Moringaceae family. The medicinal values of *M. oleifera* plant parts such as roots, bark, leaves, flowers, fruits, and seeds have been documented to have antimicrobial activity [19] [20] [21] [22], antidiabetic [23] [24], hepatoprotective [25]. Recently, hypocholesterolemic and antiobesity activity of crude extract of *M. oleifera* leaf was explored [26] [27], but whether the anti-obesity property is related to modulating the gut microbiome has not been yet investigated; hence our study was conducted to investigate the effect of aqueous extract of *M. oleifera* leaves on caecal Lactobacillus and Bifidobacteria in experimentally induced obesity.

Material and Methods

The experimental procedures were carried out in biochemistry and molecular biology labs in the national research centre and Clinilab, Cairo, Egypt, following the actual law of animal protection which was approved by the Ethical Committee of National Research Center.

Moringa oleifera leaf extract was used in this study after being collected from Pilbis, Sharqia Governorate, Egypt, and processed in the labs of Moringa Unit, National Research Centre. As powdered air dried Leaves (1 Kg) were extracted with (100 mL) of distilled water by boiling at a temperature from 80 to 100°C in reflux for 3 h to achieve an initial extract. The extract was then filtered with filter paper after cooling to room temperature. The aqueous extract stock solution (100 mg/mL) was stored at 4°C

until further use according to the method used by Lsitua et al., in 2013 [28].

Two types of diets were used in feeding a total of 45 young Swiss albino mice of either sex, (18–20 g). A normal diet has fat content 3% of energy, and a high-fat diet (HFD) has fat content 20% of energy [29]. The fat content of the HFD was beef tallow 15% and corn oil 5%. The diets have a formula covering the nutrient requirements of the mice which were divided into three groups of 15 in a completely randomized design: Group 1, Normal control group in which the animals were fed on a standard chow diet and water ad libitum; Group 2, H.F.D (obese) group in which the animals were fed on H.F.D (standard chow diet+20% beef tallow) and water ad libitum; Group 3, H.F.D+M. oleifera (treated) group, in which the animals were fed on H.F.D and received *M. oleifera* leaf extract orally along the experiment duration (200 mg/Kg).

After 3 months of treatment, all mice were anaesthetised with ether and sacrificed. The abdominal cavity was opened to expose the gastrointestinal tract. The digest of the cecum was removed immediately and were stored at -80°C until further analyses. Blood samples were collected by carotid bleeding separately into sterilised dry centrifugation tubes and allowed to stand for 30 minutes at 20–25°C. The clear serum was separated at 2500 rpm for 10 min using a centrifuge. Serum total cholesterol (Tc), serum high-density lipoprotein cholesterol (HDLc), serum triglycerides (TG), all were performed using Biodiagnostic Chemical Company kit (Egyppt) according to the instructions of the suppliers.

Serum low-density cholesterol LDL-C was calculated according to the equation, as follows:

$$\text{LDL-C} = \text{Total cholesterol} - \text{HDL-C} - (\text{Triglyceride}/5) \quad [30].$$

Serum Interleukin 6 as an inflammatory marker was performed using Enzyme Immunoassay kit (ELSA) according to the instructions of the suppliers (BIOS Company) Bacterial DNA was isolated from the digested samples using QIAamp DNA Stool Mini Kit according to the manufacturer's protocol. The pellet was suspended in 180 µl TE buffer [10 mmol/L Tris-HCl (pH 8.0), 2 mmol/L EDTA], and the mixture was briefly mixed on a vortex mixer. The suspension was incubated for 30 min at 37°C, 20 µl proteinase K and 200 µl Buffer were added. The pellets were mixed by vortexing and incubated at 56°C for 30 min and then for further 15 min at 95°C. The supernatant was transferred to a sterile tube and was stored at -20°C until PCR testing (Qi gene protocol). Quantitative real-time PCR was carried out with QuantiTect SYBR Green real-time PCR detection system (Qi gene). Two different genus-specific primer sets were used in this study, (g-Bifid-F/g-Bifid-R) for Bifidobacterium and (Lacto-16S-F/Lacto-16S-R) for Lactobacillus [31].

The 20- μ l reaction mixture contained 10 μ l of QuantiTect™ SYBR® green master mix, 1 μ l of 25 mmol l⁻¹ MgCl₂, 1 μ l (0.2%) of BSA, 2 μ l of each primer (5 mmol l⁻¹) and 4 μ l of template DNA extraction. Each PCR run included a 15-min activation time at 95°C as required by the instrument. The three-step cycle included denaturing (94°C, 15 s), annealing (60°C, 30 s) and extension (72°C, 30 s). At the end of each PCR run, melting curve analysis was performed from 72 to 95°C for detecting nonspecific PCR product and primer-dimers.

The agarose gel electrophoresis was performed to confirm further the specific PCR products. The PCR products were separated by an electrophoresis system at a constant voltage of 80V for 50 min. Then, the gel was stained with ethidium bromide (Sigma, USA) staining (0.5 μ gml⁻¹) for 5 min and followed by washing with distilled water for about 30 min. Finally, the gel was visualised under UV transilluminator (VilberLourmat, Cedex, France) and the photos were taken using a gel documentation system (Bio-Rad Gel Doc 2000 Model Imaging System).

Standards were set-up before starting quantitative sample DNA extraction by real-time PCR for quantifying the sample DNA.

Results

Our results showed the high efficiency of dietary fat in the significant ($P < 0.05$) increase of the blood lipid, IL-6 levels along with the significant ($P < 0.05$) increase in total body weight in mice fed on HFD compared to control (Table 1 & 2).

Table 1: Statistical significant differences of lipid profile and IL-6 in high-fat diet group after 3 months of feeding $P < 0.05$

Parameters	Groups	N	Min.	Max.	Mean \pm S.D.	Per cent change	P value
Cholesterol (mg/dl)	Control group	15	106	177	36.64 \pm 23.59	100%	0.001
	H.F.D. (Obese) group	15	200	247	221.79 \pm 14.38	162.31%	
Triglycerides (mg/dl)	Control group	15	65	94	82.14 \pm 9.36	100%	0.001
	H.F.D. (Obese) group	15	130	228	167.29 \pm 30.30	203.65%	
HDL (mg/dl)	Control group	15	39	80	54.29 \pm 13.47	100%	0.001
	H.F.D. (Obese) group	15	30	45	36.79 \pm 5.09	67.76%	
LDL (mg/dl)	Control group	15	46	101	70.93 \pm 18.41	100%	0.001
	H.F.D. (Obese) group	15	82	132	102.93 \pm 14.95	145.12%	
IL-6 (mg/dl)	Control group	15	2.16	7.40	3.95 \pm 1.54	100%	0.001
	H.F.D. (Obese) group	15	2.29	14.22	7.67 \pm 2.72	194.23%	

Upon high fat-diet feeding, the amount of Lactobacilli in caecal digesta was significantly ($P < 0.05$) higher than those in control group while the amount of Bifidobacteria was significantly ($P < 0.05$) lower (Table 3).

Table 2: Statistical significant differences in total body weight in the obese group after 3 months of feeding $P < 0.05$

Parameters	Groups	N	Min.	Max.	Mean \pm S.D.	P value
Total body weight (g)	Control group	15	18	21	19.5 \pm 1.20	0.001
	Obese group	15	27	35	31.0 \pm 1.11	

Table 3: Statistical significant differences of Bifidobacteria and Lactobacillus levels in the obese group after 3 months of feeding $P < 0.05$

Parameters	Groups	N	Min.	Max.	Mean \pm S.D.	Per cent Change	P value
Bifidobacteria	Control group	15	7.50	11.00	9.25 \pm 1.29	100%	0.001
	Obese group	15	3.00	6.50	4.65 \pm 1.11	50.27%	
Lactobacilli	Control group	15	7.00	10.90	8.72 \pm 1.06	100%	0.001
	Obese group	15	17.5	24.00	21.08 \pm 2.04	241.74%	

After three months of treatment with the aqueous extract of Moringa oleifera leaf, these mice showed a significant ($P < 0.05$) decrease in total body weight, Lactobacillus, blood cholesterol, triglycerides, LDLc and a significant ($P < 0.05$) increase in Bifidobacteria, HDLc compared to H.F.D (obese group). However, the decrease in IL-6 was not significant (Table 4, 5, 6).

Table 4: Statistical significant differences in biochemical parameters in obese and Moringa treated groups after 3 months of treatment with Moringa oleifera aqueous leaf extract $P < 0.05$

Parameters	Groups	N	Min.	Max.	Mean \pm S.D.	Per cent change	P value
Cholesterol (mg/dl)	H.F.D. (Obese) group	15	200	247	221.79 \pm 14.38	100%	0.001
	Moringa oleifera treated group	15	150	200	170.50 \pm 16.98	76.88%	
Triglycerides (mg/dl)	H.F.D. (Obese) group	15	130	228	167.29 \pm 30.30	100%	0.001
	Moringa oleifera treated group	15	100	150	119.50 \pm 15.79	71.43%	
HDL (mg/dl)	H.F.D. (Obese) group	15	30	45	36.79 \pm 5.09	100%	0.001
	Moringa oleifera treated group	15	45	85	63.71 \pm 11.98	173.20%	
LDL (mg/dl)	H.F.D. (Obese) group	15	82	132	102.93 \pm 14.95	100%	0.001
	Moringa oleifera treated group	15	54	120	75.86 \pm 23.33	73.70%	
IL-6 (mg/dl)	H.F.D. (Obese) group	15	2.29	14	7.67 \pm 2.72	100%	0.478
	Moringa oleifera treated group	15	3.02	11.21	6.98 \pm 2.36	90.98%	

Discussion

The significant metabolic disturbance in sort of total body weight gain, and blood lipid increase, in our high-fat diet group mice, is obviously due to the positive fat balance and consequently to adipose mass accumulation [32] [33].

Table 5: Statistical significant differences of total body weight in obese and Moringa treated groups after 3 months of treatment with Moringa oleifera aqueous leaf extract $P = 0.05$

Parameters	Groups	N	Min.	Max.	Mean \pm S.D.	P value
Total body weight (g)	Obese group	15	27	35	31 \pm 1.11	0.05
	Moringa oleifera treated group	15	26	29	28 \pm 2.3	

Energy balance not only depends on the diet but also on the microbiome as the amount of energy harvested is hypothesised to be influenced by the composition of the gut microbiota [34] [35] [36].

The HF diet group mice in this study had lower numbers of Bifidobacteria and higher numbers of Lactobacilli compared to control. Hildebrandt et al. approved in 2009 such alteration which associated with switching to the HF diet, a decrease in the Bacteroidetes level and an increase in both Firmicutes and Proteobacteria levels, in particular [37].

Table 6: Statistical significant differences of Bifidobacteria and Lactobacillus levels in obese and Moringa treated groups after 3 months of treatment with Moringa oleifera aqueous leaf extract P < 0.05

Parameters	Groups	N	Min.	Max.	Mean \pm S.D.	Per cent Change	P value
Bifidobacteria	Obese group	15	3.0	6.50	4.65 \pm 1.11	100%	0.001
	Moringa oleifera treated group	15	6.00	9.10	7.92 \pm 0.96	170.35%	
Lactobacilli	Obese group	15	17.50	24.00	21.08 \pm 2.04	100%	0.001
	Moringa oleifera treated group	15	12.50	18.00	14.73 \pm 1.65	69.86%	

Recent work that was conducted by the Turnbaugh team showed that specific enzymatic activities of obese individuals were found in Gram-positive bacteria of the Firmicutes phylum (of which Lactobacillus belong to) rather than in Bacteroidetes [38] [39] [40]. They also described a decrease in Proteobacteria and Bifidobacterium spp. Upon high fat-diet feeding [41]. Other work by Million supports that obesity is linked to enrichment in the concentrations of gut Lactobacilli, especially Lactobacilli reuteri [40] [42].

There are several possible suggested mechanisms as a causal link between gut microbiota alterations and obesity. Among them are:

1. Increasing energy recovery from the diet by gut microbiota which influences host energy storage.
2. Modulating liver lipogenesis.
3. Regulating appetite through gut satiety hormones.
4. Activating innate immunity through LPS-Toll-like receptors [43] [44].

In the framework of microbiota-related nutrition, the clear energy imbalance could affect the gut barrier through changing the intestinal wall leading to a state of inflammation; a potential condition caused an increase in blood IL-6 level shown in our high-fat feeding mice. In 2016, Agus A et al., confirmed a modification of intestinal mucosa by dysbiosis becoming thinner and more permeable to pathogens and antigens with a consequent establishment of chronic low-grade, inflammation [45].

Although attempts to use natural remedies for weight loss has increased. Very little is known about the impact of Moringa on the gut microbiome which is a significant factor in the pathogenesis of obesity. Available evidence approved that leaves of *M. oleifera* possess hypolipidemic and antiobesity effects [19] [20] [21] [22] [23] [24] [25] [26] [27]. Whether its contribution is part of improving dysbiosis associated with diet-induced obesity is poorly understood. Therefore our study was conducted to explore the link between the gut microbiota alteration and the anti-obesity effect of the Moringa leaf extract.

Our results confirmed that treatment with Moringa oleifera leaf extract was effective in reducing weight gain and the consequent metabolic disturbance in obese group mice following H.F.D

feeding. Apart from a reduction in body weight, treatment with aqueous extract of Moringa oleifera leaf was observed to attenuate the levels of total cholesterol and LDL significantly and increased the level of HDL level in mice fed on HFD. According to Farooq F et al., the hypolipidaemic effect of different medicinal plants has been related to their bioactive components [46]. A mechanism by which these compounds may decrease plasma cholesterol in our obese mice is that flavonoids that are contained in Moringa leaf extract may augment the activity of lecithin acyltransferase (LCAT), which regulates blood lipids. LCAT plays a key role in the incorporation of free cholesterol into HDL (this may increase HDL) and transferring it back to VLDL and LDL which are taken back later in liver cells [46] [47].

Moreover, mice in Moringa leaf extract treated group showed an improvement in dysbiosis by increasing Bifidobacteria level compared to those fed on HF diet. It is likely that changes in such gut resident microbe by Moringa treatment have a significant impact on another metabolic disturbance progression such as lipid levels and weight gain because Bifidobacteria can help Bacteroides degrade polysaccharides and inhibit exogenous cholesterol absorption from the small intestine [48].

In the last few decades, some researches linked the weight lowering effect of Moringa oleifera to the polyphenols present in their leaf extract. However, a more recent work added that polyphenols could modify the gut microbial composition, and had been converted by them to bioactive compounds in a two-way relationship "polyphenols \leftrightarrow microbiota" which greatly influence host health. On the one hand, the first direction Polyphenol, microbiota relationship has been investigated by Pacheco-Ordaz R and his team in very recent work in 2018. They reported that phenolic compounds inhibit the growth of some pathogenic bacteria such as *Escherichia coli*, while they promote the growth of some probiotics such as Lactobacillus. On the other hand, the reverse direction of microbiota, polyphenolic relationship, evidence showed that Bifidobacterium sp. and Lactobacillus sp. are among the few bacterial species catalyzing the metabolism of phenolics through some catabolic pathways most likely are responsible for the extensive breakdown of the polyphenolic structures into a series of low-molecular-weight phenolic metabolites that, being absorbable. The mechanism could be either through the glycan-degrading capability of Bacteroides, which is higher than Firmicutes or through the end products of colonic metabolism of polyphenols [50].

In this respect, Moringa oleifera leaf extract in our study could serve as a gut modulator in obesity-induced dysbiosis, however further studies are still needed.

Looking at the significant increase of Bifidobacteria level along with the decreased level of

IL-6 in Moringa oleifera treated group, predicts an improvement effect on the inflammatory status after treatment. This is in parallel with Cani et al., findings who used a rat obesity model to study the mechanism of inflammation associated with dysbiosis in obese mice. A significant correlation between increased levels of Bifidobacteria and reduced leakiness in the gut was observed, therefore allowing fewer LPS to translocate to the serum [51] [52].

Overall, these results support the dysbiosis state in response to a high-fat diet feeding and suggest that the response to Moringa oleifera leaf extract may be one potential approach to manage body weight and inflammation related to obesity through gut microbiota.

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