ORIGINAL ARTICLE

Study of Different Types of Gut Flora among Obese Egyptian Children

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ABSTRACT

Key words:
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Background and Objectives: *The gut microbiota plays a role in acquisition of nutrient and energy regulation. As it helps in calories extraction from ingested diet and facilitate storage of these calories in adipose tissue. Bacterial lipopolysaccharide (LPS) derived from gut microbiota acts as a trigger for chronic metabolic endotoxemia and systemic inflammation and this leads to development of obesity and insulin resistance. This study aims to identify the predominant gut flora in obese Egyptian children and its relation to the degree of obesity.* *Methodology:* *This was a case-control study included 20 obese and overweight children according to the International Obesity Task Force (IOTF) criteria. And 20 age and sex-matched healthy children with normal body mass index as control group. They were 22 males (55%) and 18 females (45%) whose mean age was 8.6±1 years. All cases that were on antibiotic in last three months were excluded. Clinical evaluation was done for all participants including full dietetic history. Full anthropometric measures and laboratory workup included stool culture for anaerobic bacteria (clostridia, bacterioids, bifidobacteria and lactobacilli) at enrollment for all participants were done.* *Results:* *There is statistical significant increase in Clostridia counts of Cases in comparison to control (p= 0.001) but, there was No statistical significant difference between the studied groups (P>0.05) with regard to Bifidobacteria, Bacteroids and lactobacilli. Also there is positive relationship between BMI and Clostridia colony count (P=0.024).* *Conclusion:* *There is association between increased gut clostridia (Firmicutes) and obesity in Egyptian children. However no relationship between type of gut flora and the degree of obesity, so determination of the role of the gut microbiota in childhood obesity may help to find new modalities for obesity management.*

INTRODUCTION

Obesity is a serious public health problem because of increased risk for emotional and physical problems among obese children. [1]

Gut microbiota is important in intestinal development, homeostasis and protection against pathogenic organisms; moreover, gut microbes are involved in metabolic reactions, with harvest of energy ingested but not digested by the host, they favour the development of intestinal microvilli, so they have trophic effects on the intestinal epithelium. [2]

The gut microbiota down regulates the intestinal expression of fasting-induced adipose factor (FIAF), which inhibits lipoprotein lipase in adipose tissues. FIAF leads to breakdown of lipoprotein-contained triacylglycerol into free fatty acids to be used by muscle and adipose tissues. [3]

During adolescence the gut microbiota is nearly consistent, meanwhile during old age dramatic changes of host physiology and dietary habits occur. Nevertheless, the dynamics and structure of an individual's gut microbiota is unique. [4]

The gut microbiota can be modulated through some dietary substances especially probiotics and prebiotics in a positive way and so they are important in the management of obesity [5]

Dysbiosis (imbalance) of gut microbiota leads to the progression of many diseases such as obesity, diabetes, non alcoholic fatty liver diseases, cancers, and also psychiatric illness. [6]

This work aims to identify the predominant gut flora type in obese Egyptian children and its relation to the degree of obesity.

METHODOLOGY

Study design and population

This was a case-control study that was conducted at the Clinical Nutrition Clinic and Outpatient Clinic, Children's Hospital, Ain Shams University.

It included 20 Obese and overweight children according to the International Obesity Task Force (IOTF) criteria during a period of 9 months.

Patients:

This study was conducted on 40 children recruited from the Clinical Nutrition Clinic and outpatient clinic of Children Hospital, Ain Shams University over a period of 9 months.

Children included in the study were 22 males (55%) and 18 females (45%) ranging between 7 and 10 years old and mean age was 8.6± 1 years.

First, 20 obese and overweight children were recruited as Group (I) which subdivided subsequently to subgroup (A) that represented Obese and subgroup (B) that represented overweight children according to the International Obesity Task Force (IOTF) criteria. [7] Then, 20 non obese children were recruited as Group (II), age and sex matched as a control group.

Children under antibiotic therapy or any drug known to alter gut flora during the last three months and Children with GIT diseases, diagnosed endocrinal or genetic cause of obesity were excluded from the study. Informed consent was obtained from the parents of children who were willing to participant in this study after approval of ethical committee.

Methods:

The enrolled patients were subjected to questionnaire aiming to reveal demographic data, and full dietetic history with special emphasis on feeding during infant period, 24 hours dietary recall and calculation of total caloric intake according to Food Composition Tables for Egypt. [8]

Anthropometric measurements were performed to all involved children and adolescents using standardized equipments, and following the recommendations of the International Biological Programme[9]. These measurements include Weight (Wt) in kilograms (Kg), Height (Ht) in centimeter (cm), waist circumference (WC) in centimeter (cm), hip circumference, mid upper arm circumference (MUC) in centimeter (cm) and triceps and subscapular skin fold thickness in millimeter (mm) using Harpenden skin fold caliper. Body mass index was calculated according to the equation: BMI= Weight in (Kg)/ Height in meter^2 (m^2) [10].

Age and sex-specific z scores for height, weight, and BMI were calculated by using year 2000 growth data from the National Center for Health Statistics, Centers for Disease Control and Prevention. This is through using the Window-based software Epi Info. Children were classified by BMI Z score categories as normal weight (BMI z score >-2 SD<+1 SD, overweight (BMI z score ≥ +1 SD<+2 SD, and obese (BMI z score ≥+2 SD) . [11]

Full physical examination was performed to exclude presence of dysmorphic chromosomal features or chronic diseases.

Parents collected Approximately 10 g of stool sample into sterile plastic containers and immediately stored in freezers at home, until brought to the laboratory. Stool samples (10g) were collected and held in a domestic refrigerator at 4C for no more than 2 hours before transportation to the laboratory ,Then it was inoculated on 19 ml of thioglycolate broth and transported immediately (less than half hour) to laboratory workers.

In the laboratory, the preweighed bottle was weighed again for determination of the exact weight of the fecal specimen the mother had collected. In this way, the number of bacteria per gram of feces could be calculated.

Isolation of anaerobic bacteria:

After homogenization on a shaker for 5 min and diluted by pipetting 0.5 ml in 9.5 ml of diluents (20 fold dilutions) by using a fresh pipette for each dilution step. Duplicate standard droplets (0.03 ml) of the last three or four dilutions were dropped on a suitable media by the method of Miles et al., 1938. [12]

Reinforced clostridial agar (Oxoid CM 151; Pharma-chemie, Haarlem, The Netherlands) supplemented with 0.5% glucose, 7.5% horse blood, and 0.03% China blue (ferri-ferrocyanide; Schmid Co., Stuttgart, Federal Republic of Germany) (RBC) was used for isolation of Bifidobacterium and Bacteroides spp. This medium does not inhibit growth of the anaerobes. Bifidobacterium spp. produce dark brown colonies and Bacteroides spp. produce blue, translucent colonies. The limit of detection with this medium is 10^4 CFU/g of feces. [13]

Clostridium spp. were isolated on sulfite-polymyxin-milk agar (SPM) containing 15 g of tryptone (Difco Laboratories, Detroit, Mich.), 10 g of yeast extract (E. Merck AG, Darmstadt, Federal Republic of Germany), 0.5 g of iron(III) citrate (Merck), and 18 g of Bacto-Agar (Difco) per 930 ml of distilled water; after sterilization and cooling of the medium to 56°C, 5 ml of filter-sterilized 5% Na_2SO_3, 10 ml of 0.1% colistin sulfate (Laboratoire.Roger Bellon, Paris, France), 4 ml of 1% neutral red (Difco), and 50 ml of sterile whole cow milk were added. This selective medium is suitable for the isolation of many Clostridium spp.and inhibits the growth of Bifidobacterium and Bacteroides spp. On SPM, clostridia strains appeared as large yellow colonies with smooth or rough surfaces. The medium permits the isolation of Clostridium spp.when they are present in amounts as low as 2×10^3 CFU/g of feces. [14]

Lactobacillus spp were isolated on Rogosa agar a modification of the medium described by Rogosa et al 1951[15], containing Peptone from casein 10 g, yeast extract 5 g D (+) glucose 20 g, potassium dihydrogen phosphate 6 g, ammonium citrate 2 g, Tween ® 80 1 g, sodium acetate 15g, magnesium sulfate 0.575 g, iron(II) sulfate 0.034 g and manganese sulfate 0.12 g per one liter of distilled water; bring to the boil to dissolve

completely. Add 1.32ml glacial acetic acid and mix thoroughly this adjust the pH to 5.5 then Heat to 90-100°C for 2-3 minutes with frequent agitation. On Rogosa Agar, lactobacilli appear as medium-sized to large, white colonies. [16]

The media were incubated at 37°C in anaerobic jars containing 90% H2, 5% C02, and 5% N2 and read after 72 h. All media were prepared weekly and kept under nitrogen at 4°C until use. [16]

The collected data was revised, coded, tabulated and introduced to a PC using Statistical package for Social Science.[17]

RESULTS

The results of this current study showed:

The mean weight of obese and overweight were 45.8±7.3 SD kg and 40.1±3.9 SD kg respectively and in control grou'p was 27.9±2.95 SD kg. BMI in obese was 25.7±2.06 kg/m^2, in overweight was 21.5±0.98 kg/m^2 and in control was 16.4± 0.47 kg/m^2 Table (1).

Mean ±SD of Clostridia counts were143±10.13, 143.5±14.60, 112.4±14.35 in obese cases, overweight cases and control respectively. (Table 2 & Figure 1)

There was statistically significant increase in Clostridia colony counts in fecal samples of Cases either obese or overweight (mean= 143×10^5 CFU/gm = 59% of total fecal anaerobes and 143.5×10^5 CFU/gm= 58.8%

of total fecal anaerobes respectively) in comparison to control (mean= 112×10^5 CFU/gm = 53.2% of total anaerobes), (P= 0.001), But no significant statistical difference detected between the 2 subgroups of cases (p= 0.243). (p>0.05) (Table 3)

Whereas, There was non significant statistical difference between all studied groups as regards Bacteroides colony count (P=0.898), Bifidobacteria colony count (P=0.923) or Lactobacilli colony count (P=0.990) (P>0.05) (Table 4)

There was ignificant positive relationship between BMI and Clostridia colony count (P=0.024) but showed non significant relation with other organisms (Table 5)

There was significant association between early weaning (before 4 month) and childhood obesity (P=0.002) (Table 6 & Figure 2)

Table 1: Weight and BMI distribution of the studied Children

Weight Kgm	Group I		Group II
	Obese (A)	Over wt (B)	Control
Mean	45.8	40.1	27.9
±SD	7.32	3.90	2.95
BMI	Group I		Group II
	Obese(A)	Over wt (B)	Control
Mean	25.7	21.5	16.4
±SD	2.06	0.98	0.47

Table 2: Quantitative & percentage ratios of anaerobic gut flora in Cases and control

Gut flora	Obese	Overweight	Control
Clostridia Mean percentage	143×10^5 CFU/gm 59.2%	143.5×10^5 CFU/gm 58.8%	112.4×10^5 CFU/gm 53.2%
Bacteroides Mean percentage	81.2×10^5 CFU/gm 33.6%	82.8×10^5 CFU/gm 33.9%	81.6×10^5 CFU/gm 38.6%
Bifidobacteria Mean percentage	14.6×10^5 CFU/gm 6%	14.9×10^5 CFU/gm 6%	14.6×10^5 CFU/gm 7%
Lactocbacelli Mean percentage	2.78×10^5 CFU/gm 1.2%	2.75×10^5 CFU/gm 1.3%	2.84×10^5 CFU/gm 1.2%

Table 3: Clostridia count of the studied Children

Clostridia	Group I (cases)		Group II (Control)
	Obese	Over wt	
Mean	143	143.5	112.4
+SD	10.13	14.60	14.35
f. test	27.114		
p. value	0.001		
Tukey' s test			
Obese & over wt	**Obese & control**		**Over wt & control**
0.243	**0.001**		**0.001**

Table 4: Bacteroides , Bifidobacteriaand Lactobacilli count of the studied Children

Bacteroides	Group I (cases)		Group II (Control)
	Obese	Over wt	
Mean	81.2	82.8	81.6
+SD	6.39	8.53	7.24
f. test	0.107		
p. value	0.898		
Tukey'stest			
Obese & over wt	Obese & control		Over wt & control
0.963	0.951		0.869
Bifidobacteria	Group I (cases)		Group II (Control)
	Obese	Over wt	
Mean	14.6	14.9	14.6
+SD	1.99	2.24	2.04
f. test	0.081		
p. value	0.923		
Tukey'stest			
Obese & over wt	Obese & control		Over wt & control
0.856	0.685		0.774
Lactobacilli	Group I (cases)		Group II Control
	Obese	Over wt	
Mean	2.78	2.75	2.84
+SD	1.66	1.68	1.48
f. test	0.011		
p. value	0.990		
Tukey'stest			
Obese & over wt	Obese & control		Over wt & control
0.751	0.639		0.885

Table 5: Correlation between BMI and Clostridia, Bacteroides,Bifidobacteria and Lactobacilli count

		Bifidobacteria	Lactobacilli	Bacteroides	Clostridia
BMI	r.	-0.140	0.043	-0.032	-0.336
	p. value	0.550	0.857	0.985	0.024*

Table (6) Shows time of weaning distribution of the studied children

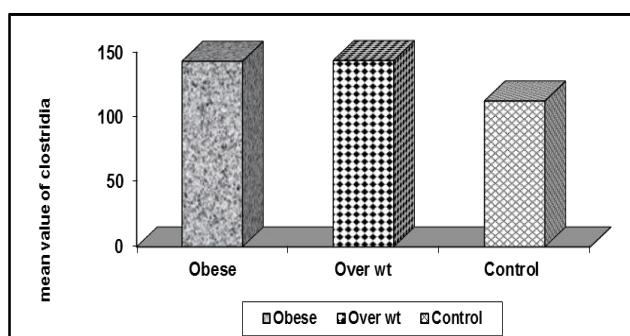| Weaning		G I	G II	Total
After 4 Months	N	5	15	20
	%	25.0%	75.0%	50.0%
Before 4 Months	N	15	5	20
	%	75.0%	25.0%	50.0%
Total	N	20	20	40
	%	100.0%	100.0%	100.0%
Chi-square	X^2	10.0		
	P-value	0.002		

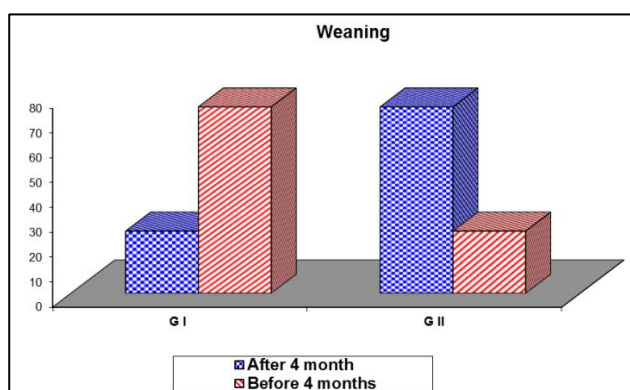**Fig. 1:** Clostridia count of the studied Children

Fig. 2: Time of weaning distribution of the studied children

DISCUSSION

The gut microbiota affects nutrient acquisition and energy regulation. Evidence suggests that the metabolic activities of the gut microbiota facilitate the extraction of calories from ingested dietary substances and help to store these calories in host adipose tissue for later use. [18]

The identification of the gut microbiota as an environmental factor that modulates host energy and lipid metabolism has revealed a novel therapeutic target to treat metabolic diseases. [9]

Using culture-dependent techniques the present study showed that

Statistically significant higher clostridia counts of cases either overweight or obese in comparison to control group (*P*=0.001), But no significant statistical difference detected between the 2 subgroups of cases (*p*= 0.243) (*p*>0.05).

These results may be explained as Clostridia could play a role in obesity together with diet by affecting host metabolism .[20] Clostridia clusters integrates the main butyrate-producing bacteria in the distal colon, These bacteria are responsible for generating butyric acid from carbohydrate fermentation, which fuels epithelial cells covering up to 70% of their energy needs [21].

Firmicutes including Clostridia could harvest energy from the diet, by accelerate fermentation of otherwise indigestable carbohydrates, thereby leading to increased acetate production and subsequently higher energy absorption which could account for up to 10–15% of our daily caloric supply [22].

Clostridia results in the present study are in agreement with the study done by Duncan et al.[23] who show significant diet-dependent reductions in a group of butyrate-producing Firmicutes (Clostridium coccoides) in fecal samples from obese subjects on weight loss diet. Although their results are close to our results, however their study related these reductions to the dietary intervention and type of food itself, not to the BMI changes.

The results of this work agree also with the study done by Ismail et al. [24] that found that distribution of Firmicutes was significantly increased in the obese group compared to the normal weight group, however they included adults with children in the study.

These results agree also with the study done by Bervoets et al.[25] and Riva et al.[26] that showed that obese children had an elevated Firmicutes-to-Bacteroidetes ratio compared to lean children.

Also Nakayama et al.[27], states that firmicutes, the order Clostridiales showed significant positive correlation with total fat intake (rho = 0.5443; CI95%: 0.28 to 0.73; *p* = 0.0003).

These results also consistent with the study done by Maksimova et al. [28] who found that the proportion of Clostridia positively correlated with BMI (r=0.56, *p*=0.006)

This study partially agrees with the conclusion of Korpela et al.[29], they concluded that Clostridial species, in particular, were indicative of the amenability of the gut microbiota to dietary modification. As they differentiated between two groups of obese with or without high clostridia sphenoides and found that the degree of benefit from dietary intervention was better in the former group.

In contrast, the results of this work as regards clostridia count disagree with the studies done by Xu et al.[30] that found no Significant changes in gut microbiota numbers between cases and control, whereas Zuo et al. [31] found that the obese group exhibited a lower amount of Clostridia in their stool than the normal weight group.

As regards Bacteroides, the present study showed no significant statistical difference in Bacteroides colony counts (*P*=0.89) in fecal samples of Cases in comparison to control.

These results are in agreement with the study done by Duncan et al. [23] detected no difference between obese and non-obese individuals in the proportion of Bacteroides measured in their fecal samples before and after dietary intervention. Also Karlsson et al. [32] revealed the same results as regards Bacteroides.

In contrast, Zuo et al.[31], Xu et al.[30] revealed that Bacteroides copy number significantly lower in the

obese group as compared to the normal group, Nadal et al.[22] showed the same results but after following dietary intervention programs.

On the other hand, Ismail et al.[24] found that BMI is positively correlated with Bacteroides proportion in the studied groups. While, Vael et al.[33] found that high Bacteroides fragilis concentration, was associated with a higher BMI SDS during the first three years of life.

As regards Bifidobacteria the present study showed no significant statistical difference in Bifidobacteria, colony counts ($P= 0.9$) in fecal samples of Cases in comparison to control.

These results are in agreement with the study done by Nadal et al.[22], Vael et al.[33] and Karlsson et al.[32] who revealed that Bifidobacteria neither significantly correlated with weight loss nor with BMI z-score reductions.

In contrast Kalliomaki et al.[34] found that Bifidobacteria significantly higher in children who remained normal weight at 7 years than in children developing overweight. Also Maksimova et al.[28] found that Bifidobacteria negatively correlated with BMI (r=-0.42, p=0.046)

On the other hand Turnbaugh et al.[35] revealed a higher proportion of phyla Actinobacteria, including bifidobacteria, among obese subjects.

As regards Lactobacilli the present study showed no significant statistical difference in Lactobacilli, colony counts ($P= 0.99$) in fecal samples of Cases in comparison to control.

These results are in agreement with the studies done by Nadal et al.[22], Vael et al.[33] and Karlsson et al.[32] as they found no difference between obese and non-obese individuals in the proportion of Lactobacilli.

In contrast Santacruz et al.[36] showed that BMI reduction in obese led to a concomitant increase in the concentrations of Lactobacillus species.

On the other hand Bervoets et al.[25], found that lactobacilli positively correlated with childhood obesity.

This study found significant positive association between early weaning (before 4 month) and childhood obesity as 75% of cases were weaned before 4th month in comparison to only 25% of control (p=0.002)

It is plausible that the introduction of specific dietary components in solid foods or resultant alterations in gut flora may result in epigenetic modification of metabolic programming; these early feeding-related changes may have lifelong detrimental effect. [37]

The previous results are supported by by Bergström et al.[38] who applied on a large Cohort of Danish Infants, they found that significant changes in the gut microbiota occurred, particularly from age 9 to 18 months, as cessation of breastfeeding and introduction of a complementary feeding may induce replacement of a microbiota (characterized by lactobacilli, bifidobacteria, and Enterobacteriaceae with a microbiota dominated by Clostridium spp. and Bacteroides spp). They also found that from 9 to 18 months, a positive correlation between the increase in body mass index and the increase of the short-chain-fatty-acid-producing clostridia was observed.

On the other hand Reilly et al.[39] found no relationship between the timing of introduction of complementary feeding and childhood obesity. Also Burdette et al.[40] found neither breastfeeding nor the timing of introduction of solid foods (before or after 4 months of age) had an effect on adiposity at age 5 years.

There is strong evidence that the gut microbiota plays an important role in the regulation of energy balance and weight in humans and may influence the development of obesity.

We concluded that an association between increased gut clostridia (Firmicutes) and obesity in Egyptian children has been identified. However no relationship between type of gut flora and the degree of obesity has been detected, so determination of the role of the gut microbiota in childhood obesity may help us to find new modalities for obesity management.

Conflict of interest:
No conflict of interests by anyone of the authors

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