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RELATION OF *LACTOBACILLI ACIDOPHILUS* TO OBESITY IN EGYPTIAN POPULATION

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Current considerations are existed about the sharing role of gut microbiota in the enhancement of obesity and allied comorbidities. This observational case-control study assessed the possible relation of *Lactobacilli acidophilus* to obesity in a sample of Egyptian population by real-time PCR in stool. The study enrolled 20 healthy slim subjects and 40 subjects who had BMI >25 kg/m^2. Routine laboratory analysis and identification of stool *L. acidophilus* by quantitative real time PCR technique was performed for all enrolled subjects. *Lactobacillus acidophilus* was expressed in 21/40 (52.5%) fecal samples of obese cases and 16/20 (80%) of fecal samples of non-obese ones. In rest of samples in both groups, the expression was below the detection limit. The results showed that the mean lactobacilli CT was expressed in obese cases (38.89±2.57) compared to (36.08±4.63) in non-obese cases, with high significant difference (P =0.04). *Lactobacillus acidophilus* was significantly lowered in obese Egyptian patients. The argument about significance correlation between imbalance in gut microbiota and obesity is one of the hot topics in medicine.

Key words: Obesity, Gut Dysbiosis, *Lactobacilli acidophilus*.

Introduction

Globally, obesity prevalence has been surged in the last 50 years, touching a pandemic state (Blüher, 2019). Surplus body weight ranked as the 6th most crucial risk factor added to entire burden of diseases worldwide with about 1.1 billion adults and 10% of children are nowadays categorized either overweight or obese (D'Ugo *et al,* 2019).

In Egypt, around 36% of the adult population was considered obese (Salman *et al,* 2018) and increasing number of diabetic patients (El-Tawdy *et al,* 2017)

Obesity heralds copious of morbidities and is also allied to higher mortality. For instance, it's is a risk factor for type 2 diabetes mellitus, elevated blood pressure, dyslipidemia, cardiovascular diseases (CVD), respiratory ailments, joint complaints, psychosocial conditions, and multitude of cancers including colon, esophagus, pancreas, breast, and prostate (Cornejo-Pareja *et al,* 2019).

Considerations are existed about the sharing role of gut microbiota in the obesity enhancement and its allied comorbidities (Al-Assal *et al,* 2018). Dysbiosis, imbalance in the microbial classes' abundance, was frequently related to gut barrier malfunction and inflammatory cell instigation (Bäckhed *et al,* 2012).

The failure to amply adjust the microbial diversity configuration occurs at the onset and chronicity of several illnesses, involving inflammatory bowel diseases (IBD), irritable bowel syndrome (IBS), diabetes, obesity and cancer (Zuo and Ng, 2018; Hills *et al,* 2019). In the early 21st century, conclusions were built on divergent gut microbiota constitution amongst lean and obese mice, which were subsequently validated in humans proposing that this configuration could influence obesity. Ever since, many studies declared an intimate relation between microbiota and obesity, postulating that gut microbiota may be fundamental to energy homeostasis regulation (Cuevas-Sierra *et al,* 2019).

Obese patients have a minor diversity and richness in bacterial component of gut microbiota than eutrophic subjects. Obese mice presented proliferated Firmicutes and declined Bacteroidetes in faeces, irrespective of diet intake. Thus, obese humans displayed an amplified Firmicutes/Bacteroidetes ratio in faecal microbiota. But, a meta-analysis did

not authorize the lower Bacteroidetes proportion in overweight persons, suggested that disparities in the gut microbiota makeup by weight or by body mass index may not be true (Al-Assal *et al,* 2018).

Furthermore, probiotics which are health beneficial microbes predominantly categorized underneath genus Lactobacillus as *L. acidophilus* and *Bifidobacterium*, which when administered in suitable quantities confer health profits to the host, and have been concerned with innumerable physiological tasks including energy homeostasis and an up-and-coming zone of research (Arora *et al,* 2012).

This study aimed to assess the possible relation of lactobacilli acidophilus to obesity in a sample of Egyptian population by real-time PCR of lactobacilli acidophilus in stool.

Subjects and Methods

The present study enrolled 60 outpatient clinics at Kafr El-sheikh General Hospital and Ain Shams University during the period from May 2017 to June 2018. They were divided accord-ing to body mass index (BMI) into two groups G1:20 healthy slim cases and G2:40 obese cases with BMI >25 kg/m^2.

Exclusion criteria: Patients with hypothyroidism, Cushing syndrome, inflammatory bowel diseases, as well as pregnant women and patients with ascites were excluded.

The selected patients were subjected to history taking and complete general and abdominal examination. BMI was calculated by using formula: BMI= weight (kg)/height (cm). Waist to hip ratio was calculated by dividing waist circumference (WC) by hip circumference (HC).

Sample collection: Morning blood samples were drawn after a 12hr fast, a part was collect-ed on EDTA tube for determination of glycated hemoglobin, extraction of RNA, and routine CBC by Sysmex the automated hematology analyzer SF-300 (Corporation, Japan). Second part was left to clot at room temperature, and serum was separated by centrifuging for 10 minutes at 3000r.p.m, for other biochemical investigations. Fresh stool samples were put in labeled clean and sterile containers. The samples collected at home or at the hospital were kept in a refrigerator at 4°C for not more than 2hr before stored in deep-froze at -70°C until use.

Laboratory measurements: Glycosylated Haemoglobin-A1c (HbA1c) was determined by cation exchange resin method using commercial kits (Intermedical, Italy). Serum cholesterol concentration, triglycerides, HDL-cholesterol concentration were determined by using Colorimetric enzymatic method using commercial assay kit (Stanbio Laboratory, USA) and the LDL concentration. ALT & AST were determined by the enzymatic colorimetric method using a Kit (Abnova). Assessment of thyroid function was done using reagents (IBL International GmbH, Germany) by solid phase sandwich-ELISA.

Identification of stool *L acidophilus* by quantitative real time PCR: DNA isolation: DNA from bacterial colony grown in Diamon Rogosa Sharp agar (MRS) broth was extracted using proteinase K protocol after centrifugation of the broth for 2min. Briefly, bacterial cells were re-suspended in 467µl of freshly prepared (50mMTris-HCl, 10Mm EDTA, pH 7.5) 30UL of 10% SDS & 3µl of 20mg/ml proteinase-k (Sigma, Aldrich). After incubation at 65°C for 45min with shaking, DNA was obtained by phenol/chloroform/isoamyl alcohol precipitated with isopropanol washed with 70% ethanol, dried by vacuum. DNA pellet was re-suspended in 25 µl sterile distilled water and stored at 20°C.

PCR primer: DNA pellet was confirmed as bacterial origin using the universal 16-S primer. F (5- AGA GTT TCC TGG CTC TS A AG-3) and R (5-ACG ACC TTG TTA GMT CGA CTT3) amplified a 1500bp fragment. Genus specific primers of Lactobacilli identification were designed on 16SrRNA. Genus specific primers, (Sigma, USA) for Lactobacilli amplified a 123bp fragment. LbL MA1-rev (5-CTC AAA ACT AAA CA AG T TTC-3) and R16-1 (50-CTT GTA CA C A CC GCC CGT CA-3).

PCR conditions and procedure: PCR reaction mixture (25µl) composed of 25pmol of each primer, 0.2mM of each dNTP, PCR buffer 50mMTris-HCl, PH value was carried out in a Touchdown Thermal Cycler (Hybaid Middlesex, UK).

Amplification program was as follows: Initial denaturation at 95˚C for 5min, followed by 30 cycles consisted of denaturation at 95ºC for 30s, annealing at 55ºC for 30s, extension at 72˚C for 30s, & 7min final extension step at 72˚C. Amplification products were subjected to electrophoresis in 1% agar gels (Electrophoresis grade, Invitrogen) in TAE buffer (40mMTris acetate, 1ml EDTA, pH8.2) followed by ethidium bromide staining (5µg/ml) and visualized under UV light.

Statistical analysis: Data were analyzed by using SPSS version 23. Shapiro-Wilks test evaluated variables distribution. Data were expressed as M±SD or median and range. Categorical data were given in percentages. Significance for difference between groups was determined by using two-tailed Student's t-test. Qualitative variables were assessed by chi-squared χ^2test. Correlations between different parameters were done using Spearman's and Pearson's correlation coefficient. Probability (P) values of ≤0.05 were considered statistically significant indicated, but P> 0.05 was considered not significant.

Results

The results were given in tables (1 & 2) and figures (1 & 2)

Table 1: Comparison of demographic and clinical characteristic of both groups at study beginning.

Variable Groups	Non-obese (n=20)	Obese patients (n=40)	P-value
Age (Yrs.)	38.8 ±9.88	38.8 ±9.88	0.261
Male/Female Percentage of Male	11/9 (55%)	14/26 (35%)	0.139
Diabetic Non-diabetic	1(5%) 19(95%)	4(10%) 36(90%)	0.656
WHR	0.902 ±0.759	0.91 ± 0.75	0.681
BMI (Kg/m2)†	23.14±1.33	23.14±1.33	<0.001***
Liver Ultrasound: Normal	16(80%)	26(65%)	0.503
Fatty	4(20%)	12(30%)	
Cirrhosis	0(0%)	2(5%)	
Clinical parameter: HbA1c (gm%)	5.49 ±0.79	5.75± 1.01	0.320
Cholesterol (mg/dl)	161.7 ± 28.67	212.9±48.06	<0.001***
Triglycerides (mg/dl)	112.55 ± 83.05	149.93 ± 84.126	0.109
Stool analysis: Normal digestion Mal-digestion	13(65%) 7(35%)	12(30%) 28(70%)	0.01**
Expression of *L. acidophilus:* Negative Positive	4(20%) 16(80%)	19(47.5%) 21(52.5%)	0.039*
CT by RT-PCR	36.08±4.63	38.89±2.57	0.04*

BMI: Body-mass index, WHR: waist to hip ratio, Data displayed as Mean ±SD unless otherwise indicated, [a]Categorical data were summarized as number (percentage), *P ≤0.05, **P ≤0.01, ***P ≤ 0.001.

Mean age of obese cases cross matched controls and number of men in each group was insignificant. Diabetic patients, and Waist and hip ratio (W/H Ratio) showed no significant difference (P=0.656 & 0.681). Body mass index (Kg/m^2) showed significant increase in obese cases compared to slim ones (38.76±5.58 vs.23.14±1.32kg/m2, p<0 001).

The obese group included twenty fatty liver subjects (30%) and two cirrhotic patients (5%) compared to 4 (20%) fatty liver cases in non-obese group, without significant difference between both groups as to US findings (P = 0.503). The HbA1c percent in obese cases as compared to controls was without significant difference (5.75±1.01 versus 4.66±2.11 respectively, P=0.320).

The present study also showed that the mean cholesterol levels were higher in obese cases (212.9±48.06mg/dl) than in controls (161.7±28.67). This increment was statically significant (t=-5.15, P<0.001). Triglycerides level did not show significant difference between obese to slim cases (149.93±84.126 vs. 112.55±83.0mg/dl) with t=-1.629, P=0.109.

Regarding stool analysis showed that (70%) of obese cases were found to suffer from mal-digestion whereas only half of this per-

cent (35%) of control subject are suffering from mal-digestion. Obese cases showed a significant increase in percentage of patient who had male digestion compared to control subjects (P=0.01). Quantification of *L. acidophilus* in feces by real-time PCR: Lactobacilli were expressed in 21/40 (52.5%) of obese cases and 16/20 (80%) of control cases, with expression was below the detection limit in both. The mean CT at which lactobacilli expressed in the obese cases was (38.89±2.57) compared to (36.08±4.63) in control cases with higher significant difference (P =0.04). Fold change in lactobacilli expression was 0.53 in obese cases compared to control ones (Tab. 2, Figs. 1-2).

Table 2: Correlation between fold change of *Lactobacillus acidophilus* expression and other parameters changes

Parameters	Fold changes in expression of Lactobacilli	
	r	P-value
Age (Years)	0.038	0.774
CT	-0.744**	<0.001
BMI (Kg/m^2)	-0.268*	0.038
WHR	-0.264*	0.041
Medical history of diabetes	-0.052	0.693
Stool analysis	-0.080	0.542
HbA1c (per gm %)	-0.052	0.691
Cholesterol (mg/dl)	-0.093	0.478
Triglycerides (mg/dl)	0.254*	0.05

Note: BMI: Body-mass index

Pearson's correlation test showed significant negative correlation between fold change of Lactobacilli expression and CT, BMI, & WHR (r=-0.744, P<0.001, r=-0.268, P=-0.038, & r=-0.264, P=0.041, respectively). Also, a significant positive correlation between expression of lactobacilli and triglyceride level was observed (r=0.254, P=0.05).

In contrast, there was no significant correlation between Lactobacilli expression and age (r=0.038, P=0.774), HbA1c (r=-0.052, P=0.691) as well as cholesterol levels (r=-0.093, P=0.478). Spearman correlation did not show significant correlation between La ctobacilli expression and medical history of diabetes & stool analysis (r=-0.052, P=0.693 & r=-0.080, P=0.542; respectively).

Discussion

The gut dysbiosis, namely dysregulation of intestinal microbiota, and increased gut per-meability led to enhanced inflammation and are commonly seen in chronic conditions such as obesity and aging (Ouyang *et al,* 2020). Besides, Obesity and diabetes were concomitantly increased in Firmicutes and lower proportion of Bacteroidetes and Proteobacteria (Greathouse *et al,* 2019).

In the present study, *Lactobacilli acidophilus* was expressed in 21/40 (52.5%) obese cases faecal samples and 16/20 (80%) in control ones. The mean cycle threshold (CT) which Lactobacilli expressed in obese cases was (38.89±2.57) compared to (36.08±4.63) in control cases. The expression of lactobacilli was statistically higher in control cases than obese cases (P =0.04). The fold change in the expression of lactobacilli was 0.53 in obese cases compared to control subjects. Pearson's correlation test did not show significant correlation between Lactobacilli expression and BMI, WHR and positive correlation with triglyceride level. Quantitative PCR was more sensitive beyond culture to detect *Lactobacillus* species (Herbel *et al,* 2013).

Lactobacilli generally comprised less than 1% of faecal bacterial media, yet the composition of the distal parts of the gastrointestinal tract was still not clear. Lactobacilli were 90% of faecal specimens by the culture and 100% by RT-PCR (Štšepetova *et al,* 2011). Also, Million *et al.* (2012a) reported that different Lactobacillus species were associated different effects on weight change that were host-specific, and must clarify the role of Lactobacillus species in the human energy harvest and weight regulation, with attention to the potential effects of commonly

marketed lactobacillus-containing probiotics on weight gain Million *et al.* (2012b) found that the *Lactobacillus* & *Bifidobacterium* genus representatives have a critical role in weight regulation as an anti-obesity effect in experimental models and humans, or as a growth-promoter effect in agriculture depended on the strains. Wu *et al*. (2015) found that supplementation with K21 *L. plantarum* restricted weight gain in DIO mice. Halawa *et al.* (2019) by PCR found significantly lower stool *L. acidophilus* count among obese diabetic patients compared to healthy controls. Thus, lactobacilli played a main role in dairy industry as utilized in cheese and yogurts manufacture. Karami *et al,* (2017) stated that probiotics such as lactobacilli prevent the development of a wide range of human and animal's pathogens. They concluded that these bacteria can be raised for the production of various kinds of food, pharmaceutical products, and functional foods. Yogurt inclosing Lactobacillus strains provoke resistance to body weight gain induced by diet- and increased plasma cholesterol and triglyceride levels. Although several potential mechanisms have been proposed, how lactobacilli reduce serum cholesterol levels has not been well established. One of the postulated mechanisms is the assimilation of cholesterol by *L. acidophilus* as some lactobacilli, as *Lactobacillus* species, *Bifidobacterium longum*, *Clostridium perfringens,* and Bacteroides fragilis fragilis, stimulated bile salt hydrolase; in turn facilitated the de-conjugation of bile acid salts, catalysed hydrolysis of glycine- or taurine-conjugated bile salts into Amino acid residue and free bile salts and hindered entero-hepatic circulation of bile salts. This decreased serum cholesterol concentrations as cholesterol is the precursor for the de novo production of fresh bile acids. Added mechanism by which cholesterol is decreased is the production of short-chain FA (SCF) by probiotics upon fermentation, which inhibited hepatic cholesterol synthesis (Song *et al,* 2015).

Štšepetova *et al*. (2011) found great proportions of intestinal lactobacilli in elderly persons, influenced by diet type were in those (70-73%) who consumed western diet, while in a vegetarian-type diet, an upper lactobacilli prevalence was measured

Lê *et al.* (2013) assessed the relationship between lactobacilli and gut inflammation allied to obesity found that *Lactobacillus* levels did not correlate to plasma C-reactive proteins concentrations. They hypothesized that such organisms in situ provoke inflammation or other cytokines that they did not estimate, such as TNF-α or IL-6

Armougom *et al.* (2009) by real-time PCR found that the expression profiles Firmicutes concentration in obese and controls were the same. They concluded that an opposite firmicutes/bacteroidetes ratio only reduced bacteroid levels in obese.

However, Duncan *et al.* (2008) found no significant relationship between BMI or absolute weight loss and the relative bulky population of the top most groups of human colonic bacteria, comprising bacteroidetes in obese and non-obese persons. The Bacteroidetes and Clostridia cluster XIV (Firmicutes) quantities analysed by qPCR and FISH modalities, did not show correlation between altered microbiota structure & BMI, and thus people ingesting high-fat diet had lesser Clostridia, while on consuming a fiber-rich food there was an upsurge of lactic acid bacteria levels (Abenavoli *et al,* 2019).

Patil *et al.* (2012), by comparative analysis and quantification of dominant gut microbiota among lean, normal, obese and surgically-treated obese individuals of Indian origin by 16S rRNA sequencing, found no evident in distribution of the predominant bacterial phyla (Bacteroidetes & Firmicutes) with an even of Bacteroides species at genus level in obese cases with positive RT-PCR correlation between Bacteroides and BMI The unique populations with dissimilar particular habits of life, or genetic and socioeconomic status can clarify these contradictory results.

Conclusion

The argument about the gut microbiota alterations in obesity is still on-going and it may be affected by diet and many other factors. Lactobacilli are good candidate for further investigation with in vivo studies to determine its potential health profits.

Lactobacillus acidophilus was significantly lowered in the obese cases. Modulation of gut microbiota with probiotics, prebiotics, antibiotics, or other therapeutic interventions may be beneficial in obesity control.

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Explanation of figures

Fig.1: Frequencies and percentages of fecal samples showed positive expression of Lactobacilli among controls and obese cases.

Fig.2: Comparison between relative Lactobacilli expression levels in groups by RT-PCR. Lactobacilli expression levels were significantly down-regulated in obese cases compared to slim controls.