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# Quantitative of Intestinal Microbial *Lactobacillus* and *Bacteroides* Groups and the Prevalence of their Selected Species among Egyptian Obese Subjects.

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**ABSTRACT**

Obesity and alteration in human gut microbiota have been shown strong impact relation according to scientific data and WHO reports. In this study we have quantitative two bacterial groups; *Bacteroides*, and *Lactobacillus* within the fecal samples of the Egyptian obese adults and children subjects compared to normal subjects by quantitative real time PCR each group included 25 subjects. The results clearly indicated no significant difference was observed in *Bacteroides* and *Lactobacillus* groups compared to control within the obese adults group. While the results of obese children group revealed no significant difference in *Bacteroides*, and non-significant decrease in *Lactobacillus.* Further analysis of their fecal microbiota with focus on the distribution of selected strains within these two species was done by PCR using specific-species primers to find out which species could be associated with obesity. The results showed low prevalence of the all tested *Bacteroides* spp and some of *Lactobacillus* spp (*L. casei*, *L. plantarum*, *L. rhamnosus* and *L. gasseri*) in fecal microbiota of both obese groups. While high prevalence of *L. acidophilus*, *L. fermentum*, *L. reuteri* was shown in obese groups. As a conclusion certain species of *Lactobacillus* and *Bacteroides* may present cofactor for changes between lean and obesity. As the composition of gut microbiota has large interpersonal variation further investigations in this era are recommended which should also involve the bacterial genes and expressed proteins in each status. However further experiments on mice models are in going in our lab.

**Keywords:** Obesity, Gut microbiota, *Lactobacillus*, *Bacteroides*.

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## INTRODUCTION

Obesity is a growing widespread problem worldwide [1]. It is consider a major health problem because of its serious health consequences, including type 2 diabetes mellitus, cardiovascular diseases, pulmonary hypertension, obstructive sleep apnea, gastro esophageal reflux disease, musculoskeletal disorders, a variety of cancers, and a number of psychosocial concerns [2, 3]. The traditional diagnosis and treatment of obesity has been concerned of the dietary excesses or on host genes [4]. Now it is well documented that the microorganisms which colonize human body have a complex role not only maintain the host health [5] but also they could involve in disease [6, 7]. The relationship between obesity and gut microbiota composition was discovered since thirty years ago where this relationship was quite obvious after the treatment for either weight loss or gain [8, 9]. More recent evidence for contribution of the gut microbiota to obesity was reported by many researchers [10-14]. Many bacterial species have been implicated in the metabolism of dietary fibre to SCFA which consider as an important energy source for human [15-16] and prevents the accumulation of metabolic by-products, such as D-lactate [17, 18]. Furthermore, the microbiota has the ability to digest the polysaccharides to absorbable monosaccharides. In addition, they showed the ability to suppress the inhibition of lipoprotein lipase (LPL) which subsequently influence fatty acid uptake [10]. Many studies showed alterations in the composition of Firmicutes and Bacteroidetes in obese patients [19-21]. Many researchers concluded that the link between obesity and the microbiota is such a complicated status rather than just the ratio between the two phylum *Bacteroidetes* : *Firmicutes* [22, 23]. Several studies revealed the impact of certain species in body weight; Bervoets et al. (2013) [21] showed that the fecal microbiota of obese children has low percentage of *B. vulgatus* compared to lean children. While Furet et al. (2010) [24] noticed the decrease percentage of probiotic bacterium *Faecalibacterium prausnitzii* in obese and diabetic patients and this percentage was increased after doing a gastric bypass surgery to these patients. Armougom et al. (2009) [25] revealed the association of *Lactobacillus* species to obese profile such as (*L. reuteri*) and other species to lean profile such as (*L.gasseri* and *L.plantarum*).

The aim of this research work is to analyze the focal microbial composition in respect to *Lactobacillus* and *Bacteroides* groups and the prevalence of selected *Lactobacillus* sp. and *Bacteroides* sp. among obese subjects compared to normal subjects. Thus would provide data for understanding the role of microbiota in obesity and hence provide directions for control this disease.

## MATERIALS AND METHODS

### Subjects

A total number of 50 children and 50 adults were recruited from Pediatrics Clinic. Children were 25 normal weight and 25 obese, with age range from 10 years to 18 years old. While adults were 25 normal weight and 25 obese adults, with age range from 19 years to 45 years old. All subjects received antibiotic therapy within the last two months was excluded. The study protocol was approved by the Human Ethics Committee of our institution, and written informed consent was obtained.

### Human fecal samples

Fecal samples were collected from obese and lean subjects based on body mass index (obese >30 kg/m2 and non-obese <25 kg/m2).Two gm. stool from each subject were homogenized in 10 ml of phosphate buffer (pH 7) and immediately frozen at – 80°C.

### Bacterial strains and cultivation

Bacterial strains used as reference and for standard curves were either purchased from American Type Collection Culture (ATCC) or obtained from our laboratory collection. They are listed as follows: *L. rhamnosus* (ATCC 7469), *L. delbrueckii* (ATCC 9649), *L. acidophilus* (ATCC 4356),*L. plantarum* (ATCC8014),*L. reuteri*(ATCC53608),*L. paracasei* (ATCC25302), *L. fermentum* (NM213),*L. brevis*(ATCC14869), *L. gasseri*(ATCC33323), *L. casei*(NM512), *B. eggerthii* (ATCC27754), *B. fragilis* (ATCC2283), *B. ovatus* (ATCC8483), *B. uniformis*(ATCC8492), *B. thetaiotaomicron*(ATCC29148) and *B. vulgatus* (ATCC8482). Each bacterium was grown on the appropriate media and conditions.

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**Bacterial isolations**

Serial dilutions of fecal samples were incubated on four different media as described by Mitsuoka et al. (1965) [26]; for Bacteroidaseae Neomycin Brilliant Green Taurocolic acid (NBGT) agar plates, for *Lactobacillus*de Man, Rogosa and Sharpe (MRS) agar plates, Columbia blood agar (CBA) for *Clostridium* spp., and nutrient agar for others. The agar plates were incubated at 37°C for 48 h anaerobically using anaerobic jar and AnaeroGen (Oxoid)and number of colonies was randomly picked for further analysis.

**DNA Extraction**

Genomic DNA was extracted from fecal samples and pure cultures using the QIAamp DNA Stool Kit (Qiagen, Hilden, Germany)and AxyPrep bacterial genomic DNA miniprep kit (Axygen Biosciences, Union City, CA, USA) respectively according to the manufacturer's instructions.

**Quantitative real time PCR**

Quantitative real time PCR (qRT-PCR) was done using DyNAmo™ Flash Probe qPCR Kit and implemented in a PikoReal real-time PCR (Thermo Scientific). Primers and probes used (listed in Table 1) were order from sigma scientific service, Cairo, Egypt. The Ct values of 10-fold serial dilutions of each target bacterial DNA sample were determined.

**Table 1: List of the specific primers and probes used for detection *Bacteroides* and *Lactobacillus* species. (Probes are presented in bold)**

| Primer name | Primer sequence (5 3) | Target species | Reference |
|---|---|---|---|
| F_Bacter 11<br>R_Bacter 08<br>**P_Bac303** | CCT WCG ATG GAT AGG GGT T<br>CAC GCT ACT TGG CTG GTT CAG<br>**VIC-AAG GTC CCC CAC ATT G** | *Bacteroides*spp. | [27]<br>[28] |
| F_alllact_IS<br>R_alllact_IS<br>**P_alllact_IS** | TGG ATG CCT TGG CAC TAG GA<br>AAA TCT CCG GAT CAA AGC TTA CTT AT<br>**VIC-TAT TAG TTC CGT CCT TCA TC** | *Lactobacillus*spp | [29] |
| BaEGG-F<br>BaEGG-R | GTTTTTCCGCATGGTTTCAC<br>TTCACAACTGACTTAAGCAC | *B. eggerthii*<br>422bp | [30] |
| BaTHE-F<br>BaTHE-R | CCCGATGGTATAATCAGAC<br>CACAACTGACTTAACTGTCC | *B. thetaiotaomicron*<br>431bp | |
| BaUNI-F<br>BaUNI-R | TATCCAACCTGCCGATG<br>CACAACTGACTTAAGCGT | *B. uniformis*<br>482 bp | |
| BaVUL-F<br>BaVUL-R | AACCTGCCGTCTACTCTT<br>CAACTGACTTAAACATCCAT | *B. vulgatus*<br>486 bp | |
| BaOVA-F<br>BaOVA-R | 5'AAGTCGAGGGGCAGCATTTT'3<br>5'CACAACTGACTTAACAATCC'3 | *B. ovatus*<br>550 bp | |
| BaFRA-F<br>BaFRA-R | 5'AATGATTCCGCATGG TTT CA'3<br>5'ATTTTGGGATTAGCATACGG'3 | *B. fragilis*<br>1079 bp | |
| LgasseriF<br>LgasseriR | 5'TCGAGCGAGCTTGCCTAGATGAA<br>CGCGGCGTTGCTCCATCAGA | *L. gasseri*<br>372 bp | [31] |
| LfermentumF<br>LfermentumR | GCACCTGATTGATTTTGGTCG<br>GTCCATTGTGGAAGATTCCC | *L. fermentum*<br>317 bp | |
| LparacaseiF<br>LparacaseiR | CTAGCGGGTGCACTTTGTT<br>GGCCAGCTATGTATTCACTGA | *L. paracasei*<br>312 bp | |

| | | | |
|---|---|---|---|
| LdelbrueckiiF<br>LdelbrueckiiR | GGRTGATTTGTTGGACGCTAG<br>GCCGCCTTTCAAACTTGAATC | *L. delbrueckii*<br>138 bp | |
| FcaseIS<br>RcaseIS | CTATAAGTAAGCTTTGATCCGGAGATT T<br>CTTCCTGCGGGTACTGAGATGT | *L. casei*<br>132bp | [29] |
| LacidoF<br>LacidoR | TGCAAAGTGGTAGCGTAAGC<br>CCTTTCCCTCACGGTACTG | *L. acidophilus*<br>210 bp | [32] |
| LplantarumF<br>LplantarumR | ATTCATAGTCTAGTTGGAGGT<br>CCTGAACTGAGAGAATTTGA | *L. plantarum*<br>248bp | |
| LreuteriF<br>LreuteriR | GGCGGCTGTCTGGTCTGCAA<br>GCTTGCGACTCGTTGTACCGTC | *L. reuteri*<br>303 bp | |
| LbrevisF<br>LbrevisR | CTTCTGGATGATCCCGCGGCG<br>ACCGCCTGCGCTCGCTTTAC | *L. brevis*<br>369 bp | [33] |
| LrhamnosusF<br>LrhamnosusR | TGCTTGCATCTTGATTTAATTTTG<br>GGTTCTTGGATYTATGCGGTATTAG | *L. rhamnosus*<br>122 bp | [34] |

**PCR reactions**

The primers used in this work were purchased from Sigma Scientific Services, Cairo, Egypt. PCR amplifications were performed using the PCR machineMj Mini (Bio Rad, Hercules, CA, USA) using PCR Master Mix (Fermentas Life Sciences, Vilnius, Lithuania).

**Amplification of bacterial 16S rRNA genes**

Identification of selected colonies on the molecular basis at the species level was done using universal primers for amplification of 16S rRNA genesas described previously [35].

**Sequencing and identification of the amplified 16S rRNA genes**

Each amplified 16S rRNA gene was purified by QIA quick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Then it was sequenced by Lab technology analytical services, Egypt and similarity was detected using NCBI [36].

**PCR for detection of *Bacteroides and Lactobacillus* species**

The genomic DNA extracted from fecal samples of obese and control subjects was subjectedfor detection the selected species of *Bacteroides* and *Lactobacillus* using species-specific primers (Table 1) by PCR reactions as described previously. The targeted species were indicated in Table (1) the amplified products were detected by electrophoresis on 1.5% agarose gel. The size of PCR products was compared with the expected size for each strain. The quantity of selected species establishing the subject and their prevalence in each subject was estimated.

## RESULTS AND DISCUSSION

**Molecular identification of randomly selected isolates from plating fecal samples**

For a trial to get initial view about the microbial composition of the obese and lean subjects, randomly isolates from the individuals have been selected and identified. The twenty five fecal samples from each group were inoculated into four different media as indicated in the method section from which random colonies were picked. The chromosomal DNA of each selected colony was extracted and its 16s rRNA was amplified by PCR. Subsequently each amplified 16s rRNA fragment was sequenced and identified according to the homology

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search in the gene bank data base. Figures (1) and (2) showed the bacterial species that randomly detected from obese children and obese adults and their control respectively.

**Quantitative composition of the *Bacteroides* and *Lactobacillus* groups in the fecal samples of obese cases**

qRT-PCR was used to determine the difference of each *Bacteroides* and *Lactobacillus* groups between the obese and normal subjects (Figure 3). The results revealed that the number of subjects that expressed high *Bacteroides* $\geq 10^4$ was shown in the control adults and children groups. In the obese cases this percentage was slightly decreased where the number of subjects that expressed low *Bacteroides* $\leq 10^4$ was shown in the obese adults and children groups. In respect to Lactobacillus groups, the number of subjects that expressed high *Lactobacillus* $\geq 10^3$ was shown in the control adults and obese children groups, while the number of subjects that expressed low *Lactobacillus* $\leq 10^3$ was shown in the obese adults and control children groups expressed.

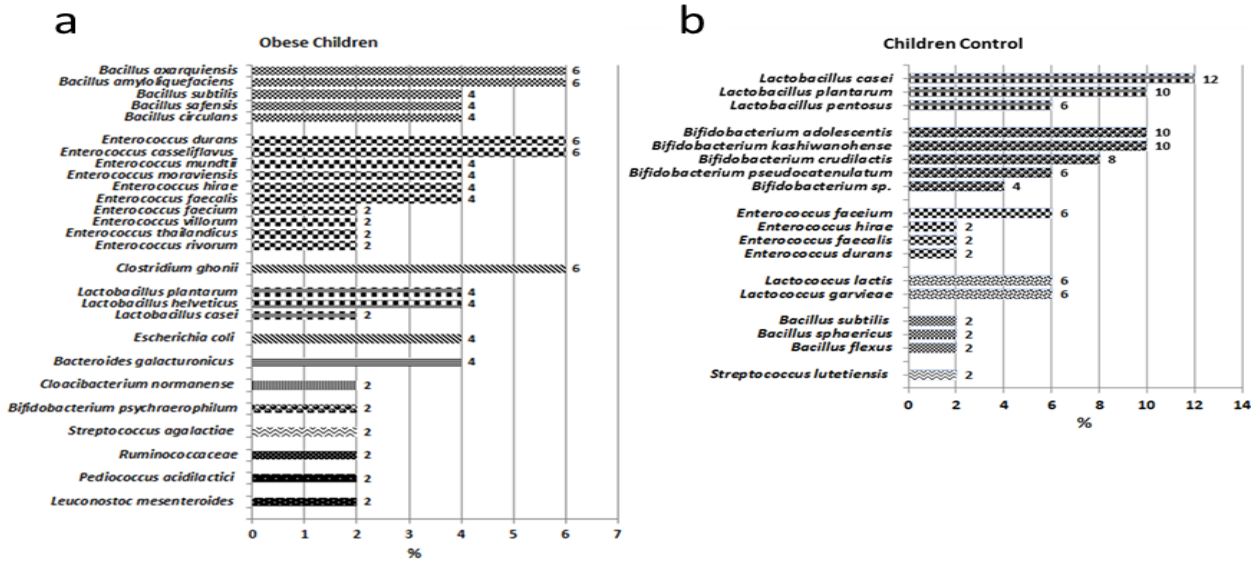


**Figure 1: Bacterial species as randomly isolated from fecal Egyptian children; obese (a) and control (b). The identification was done by amplification and sequencing of the 16s r RNA gene from the isolates.**

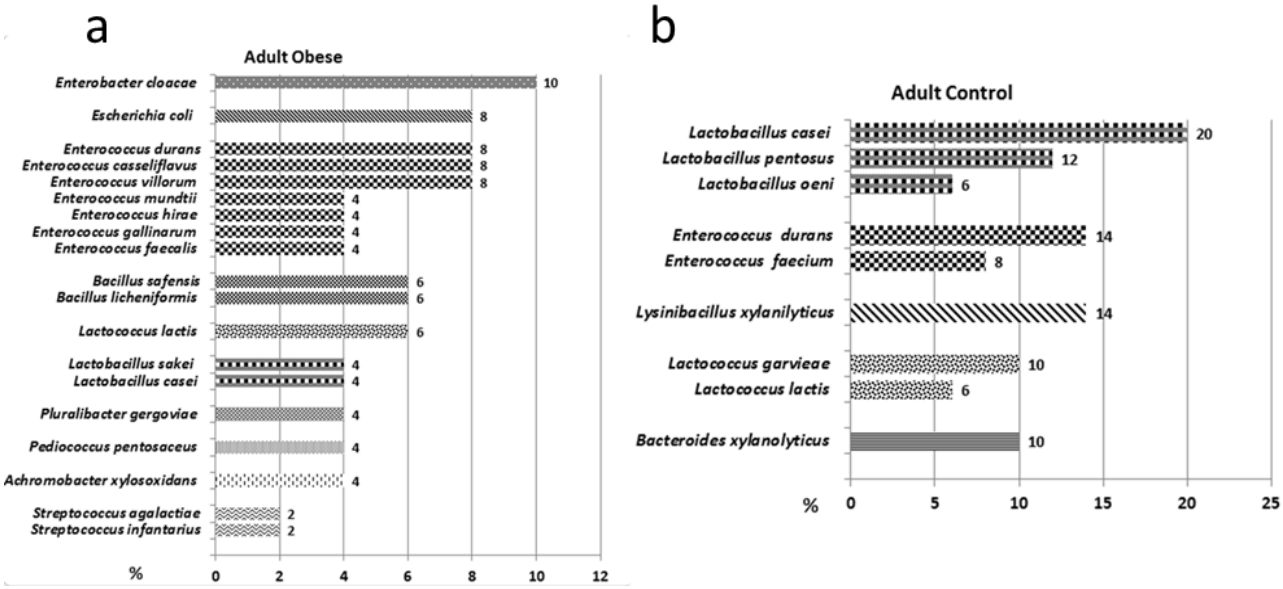


**Figure 2: Bacterial species as randomly isolated from fecal Egyptian adults; obese (a) and control (b). The identification was done by amplification and sequencing of the 16s r RNA gene from the isolates.**

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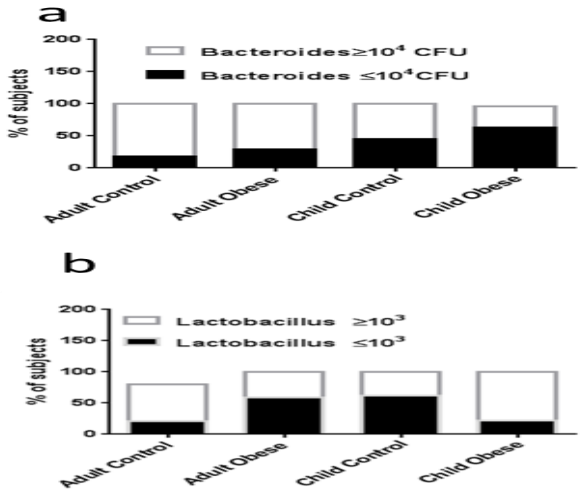


**Figure 3: Distribution of the bacterial concentration for four groups within the fecal samples of Egyptian obese adults and children compared to their control. The data presented as % for each group.**

**Analysis the fecal DNA samples for the prevalence of the *Bacteroides* species**

The distribution of six dominant *Bacteroides* species was investigated within the two obese groups(adults and children) using the species-specific primers. Each set of primers is specific for each *Bacteroides* species which amplified a specific band at the predicted size by PCR as shown in Figure (4) using the references strains. The fecal DNA samples from25 obese adults, 25 control adults, 25 obese children, and 25 control children were used as template in the PCR reactions for detection the targeted *Bacteroides* species. The sample which gave the amplified fragment as the expected size was considered positive. The results in Table (2) showed a significant variation in the prevalence of the *Bacteroides* species between the obese and control in the both cases adults and children. All the six *Bacteroides* species were presented at low percentage in obese subjects while shown high percentage in control ones.

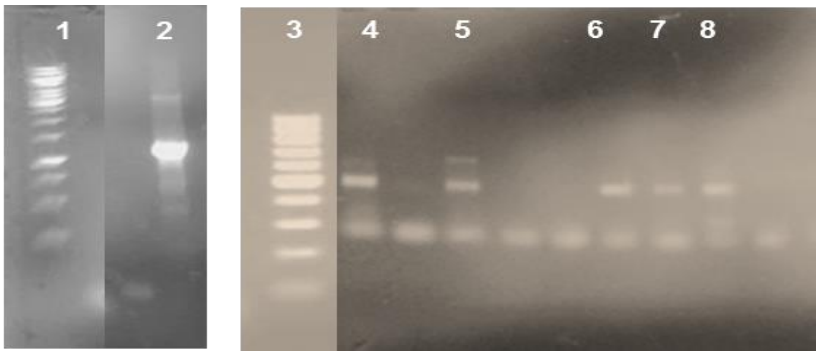


**Figure 4: Gel electrophoresis of the amplified PCR products from *Bacteroides* reference species using species-specific primers. Lanes 1: DNA ladder 1 kb; 2: *B. fragilis*; 3: 100 bp ladder; 4: *B. ovatus*; 5: *B. vulgatus*; 6: *B. uniformis*; 7: *B. thetaiotaomicron*; 8: *B. eggerthii*.**

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**Table 2: The presence number (n) and % of each *Bacteroides* species as detected by PCR specific-species primers in fecal samples of Egyptian obese and control subjects.**

| *Bacteroides* species | Children | | | | Adult | | | |
|---|---|---|---|---|---|---|---|---|
| | Obese n=25 | | Control n=25 | | Obese n=25 | | Control n=25 | |
| | n | % | n | % | n | % | n | % |
| ***B. eggerthii*** | 5 | 20 | 5 | 20 | 6 | 24 | 15 | 60 |
| ***B. fragilis*** | 7 | 28 | 21 | 84 | 8 | 32 | 18 | 72 |
| ***B. ovatus*** | 3 | 12 | 20 | 80 | 5 | 20 | 12 | 48 |
| ***B. uniformis*** | 3 | 12 | 15 | 60 | 5 | 20 | 12 | 48 |
| ***B. thetaiotaomicron*** | 5 | 20 | 18 | 72 | 0 | 0 | 8 | 32 |
| ***B. vulgatus*** | 3 | 12 | 20 | 80 | 3 | 12 | 18 | 72 |

**Table 3: The presence number (n) and % of each *Lactobacillus* species as detected by PCR specific-species primers in fecal samples of Egyptian obese and control subjects.**

| *Lactobacillus* species | Children | | | | Adult | | | |
|---|---|---|---|---|---|---|---|---|
| | Obese n= 25 | | Control n= 25 | | Obese n= 25 | | Control n= 25 | |
| | n | % | n | % | n | % | n | % |
| ***Lb. acidophilus*** | 22 | 88 | 4 | 16 | 24 | 96 | 5 | 20 |
| ***Lb. casei*** | 5 | 20 | 21 | 84 | 6 | 24 | 24 | 96 |
| ***Lb. delbrueckii*** | 12 | 48 | 16 | 64 | 10 | 40 | 17 | 68 |
| ***Lb. fermentum*** | 24 | 96 | 5 | 20 | 19 | 76 | 4 | 16 |
| ***Lb. paracasei*** | 16 | 64 | 18 | 72 | 21 | 84 | 3 | 12 |
| ***Lb. plantarum*** | 3 | 12 | 22 | 88 | 4 | 16 | 24 | 96 |
| ***Lb. reuteri*** | 25 | 100 | 2 | 8 | 23 | 92 | 3 | 12 |
| ***Lb. rhamnosus*** | 4 | 16 | 23 | 92 | 3 | 12 | 21 | 84 |
| ***Lb. helveticus*** | 22 | 88 | 3 | 12 | 3 | 12 | 24 | 96 |
| ***Lb. gasseri*** | 1 | 4 | 21 | 84 | 2 | 8 | 23 | 92 |

**Analysis of fecal DNA samples for the *Lactobacillus* species.**

The same fecal DNA samples were analyzed for their composition of the selected *Lactobacillus* species by PCR using the *Lactobacillus* specific-species primers as described in the Method section. Figure (5) show the amplified product for each species with the references *Lactobacillus species*. The sample which gave the expected band was considered positive. The results in Table (3) showed clearly the variations in the presence of the ten *Lactobacillus* species between the obese and control subjects. In the obese children; the species *Lb. acidophilus*, *L. fermentum*, *L. reuteri*, *L. brevis* exhibited high prevalence while *L. casei*, *L. plantarum*, *L.*

*rhamnosus*, *L. gasseri* exhibited low prevalence. In the other hand *L. delbrueckii* and *L. paracasei* showed moderate prevalence with both the obese and control children with no significant differences between them. In the adult group as shown in Table (3) the species *L. acidophilus*, *L. fermentum, L. paracasei*, and *L. reuteri* showed high prevalence in obese subjects and low prevalence in control subjects while *L. casei*, *L. plantarum, L. rhamnosus*, *L. brevis,* and *L. gasseri* showed very low prevalence in obese subjects. Again *Lb. delbrueckii* showed moderate distribution within the obese and control with low variations between them. The results are in agreement with other researchers as they showed that some species of *Lactobacillus* are associated with obesity and other species could act as anti-obesity candidates [25].

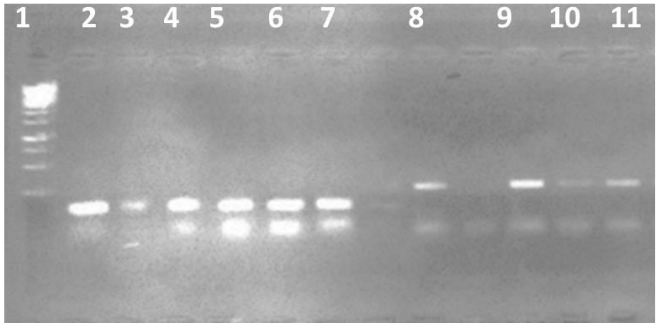


**Figure 5: Gel electrophoresis of the amplified PCR products from Lactobacillus reference species using specific-species primers. Lanes 1: DNA ladder 100 bp; 2: *L rhamnosus*; 2: *L. casei*; 3: *L. delbrueckii*; 4: *L. acidophilus*; 5: *L. plantarum*; 6: *L. reuteri*; 7: *L. paracasei*; 8: *L. fermentum*; 9: *L. brevis*; *L. gasseri*.**

It is now well documented that the gut microbiota has impact on the host weight and could control his status towards obesity. Ismail et al. (2014) and (2015) [37, 38] used the qPCR to analyze the changes in the microbial compositions in obese Egyptian children and adults compared to normal subjects respectively. The authors indicated a significant decrease in gut Bifidobacteria within the obese children while they showed reduction in gut Bifidobacteria and a significant increase in gut Enterobacteria within the obese adults. In this work we aimed to follow the changes in the *Bacteroides* and *Lactobacillus* groups among the obese children and adults compared to normal ones by qPCR using specific probes. *Bacteroides* and *Lactobacillus* bacteria are important species in the human gut for their ability to break down carbohydrates and potential toxins from plant sources in addition to their contribution in human digestion.

Preliminarily screen for the fecal samples for each group was implemented by cultivation in different media and random isolation of colonies which were identified using 16S rRNA sequencing. These random results did not provide any abnormalities in the gut microbiota between the two groups. Of course a complete analysis for the total microbial contents is required to give a clear sharp conclusion.

Although the results obtained from the qRT-PCR for quantitative the entire two groups showed no significant differences between the obese and normal cases either in children or adults but the determination of the prevalence with selected species within these groups showed significant different. The targeted certain species from *Bacteroides* and *Lactobacillus* were detected by PCR using specific-species primers as easy, fast an inexpensive method. The results showed agreement with the observed conclusion from several researches that some certain species could be associated to obesity and others could be associated to lean or weight loss.

In this work *Bacteriodes* species showed that all the tested six species expressed low levels in the fecal flora of obese children and adults in contrast to their high level in the control fecal flora. Except from that the *B. eggerthii* species which showed lower levels in children control samples as well. Our finding is in agreement with other researcher's results where Bervoets et al. (2013) [21] found low prevalence of *B. vulgatus* and high prevalence of *Lactobacillus* spp. in feces of obese children and adolescents.

Furthermore our results showed an association between obesity and lower levels of *L. casei*, *L. plantarum*, *L. rhamnosus*and *L. gasseri* in fecal microbiota of both obese children and adults. In addition to these species *L. brevis* showed lower levels in obese adults while showed high levels in obese children. In the other

hand *L. acidophilus*, *L. fermentum*, *L. reuteri* showed high levels in fecal flora of both obese children and adults. Our results are similar to Million et al (2012a) [39] where they showed high levels of *L. reuteri* in human obesity gut microbiota while low levels of *L. casei*/*paracasei* and *L. plantarum* except *L. paracasei* in our results showed different pattern. Million et al (2012b) [40] conducted an *in vivo* experimental in mice and human to explore the relation between the obesity and *Lactobacillus* species, their results showed that L. fermentum was associated with weight gain in animals while *L. plantarum* was associated with weight loss in animals and *L. gasseri* was associated with weight loss both in obese humans and in animals. Kang et al. (2013) [41] showed significant reduction in the body weight of mice groups after administration of *L. gasseri* BNR17 strain. The authors suggested the anti-obesity actions of *L. gasseri* BNR17 strain could be referred to increased expression level of the main glucose transporter-4 gene and decreased insulin levels. While Lee HY et al. (2006) [42] and Lee K et al. (2007) [43] explained the anti-obesity actions of *L. rhamnosus* and *L. plantarum* could be due to the production of conjugated linoleic acid (CLA) which play role in increasing energy expenditure. Talarico et al. (1988) [44] showed that *L. reuteri* administration to pigs, turkeys and rats caused weight increase but they could not illustrate the exact mechanism for this action. Bäckhed et al.(2007)[45] mentioned the ability of gut microbiota for hydrolysis of polysaccharides to monosaccharides and activating lipoprotein lipase causing rapid absorbance of glucose and increasing serum glucose and insulin. They also proposed the ability of certain composition of gut microbiota to control the circulating lipoprotein lipase inhibitor and regulator of peripheral lipid and glucose metabolism. Bäckhed et al. (2004) [46] suggested that the pro- or anti-inflammatory properties of some bacterial species could regulate the fat storage in the host. However the actual impact of these bacterial species on obesity and weight gain still need more research to unravel this correlation and pointed the mechanisms behind. In the mean while our ongoing step would be *in vivo* experiments on mice models resembles to human microbiota to investigate the role of certain species in weight gain or loss and addressing the possibility of selected probiotic strains which isolated by our group to control the obesity and its complicated.

## CONCLUSION

Our initial screening confirmed the association of certain species and weight gain or loss within the microbiota of Egyptian obese children and adults. This data would serve in using specific strains in faecal transplant as therapeutic strategy for obesity.

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