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## Original Research Article

## Human gut microbiota as a potential source to treat obesity

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## ABSTRACT

**Background:** The human gut microbiome is crucial to human health, illness, and diseases. These bacteria support the host system in a number of ways. Nonetheless, it appears that the species composition and alterations in the gut microbiota contribute to obesity. Screening of potential gut microbiota is inevitable at this point to address multiple health issues in the field of medical microbiology.

**Aim & Objective:** Characterizing the gut microbiota of healthy individuals and determining the presence of inclinations to degrade cholesterol, which may aid in the treatment of obesity, were the objectives of the study.

**Materials and Methods:** For this investigation 30 isolates were isolated from the faecal samples of the selected healthy individuals. On the basis of the morphological and biochemical features, 17 out of 30 isolates were selected for further studies. Based on colony morphology and biochemical tests 8 isolates were selected for screening of cholesterol degradation and lecithinase enzyme production. The ability of the selected isolates to withstand acidic pH was then examined throughout a variety of pH values. Additionally, their ability to withstand bile salts at various oxbile concentrations was examined. Based on the isolates' results, which demonstrate strong functional properties, 16s rRNA sequencing was used as a molecular methodology to identify them.

**Results:** The study's findings showed that the majority of the isolates that were chosen were non-motile, urease negative, catalase and oxidase negative. Lecithinase production was also negative in every isolate. Among the selected isolates 8 of them were selected for screening of cholesterol degradation. Two of the eight isolates that were chosen demonstrated the ability to degrade cholesterol, as evidenced by their production of two short chain fatty acids such as butyric and lactic acids. The ability to withstand different pH and bile concentrations were examined. High bile concentrations and low acid pH were conditions that both isolates could withstand and develop in. Based on the isolates' results, which demonstrate strong functional properties, 16s rRNA sequencing was used as a molecular methodology to identify them. It was determined that the isolates were *Enterococcus faecium* MN294515 and *Enterococcus faecium* MN535170.

**Conclusion:** The majority of the cultures found in this investigation were gram positive bacteria. The two isolates of *Enterococcus faecium* MN294515 and *Enterococcus faecium* MN535170 can be further studied to determine which probiotic is more effective in treating obesity, a long-standing condition.

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## 1. Introduction

The Gastrointestinal tract (GIT) of the human is mainly composed of several number of microbial communities

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with a mutualistic relationship.<sup>1-3</sup> Gut microbes involved in an improvement of function of immune system and vulnerability to disease in human. The concentration of the gut microbiota varies from  $10^{11}$ - $10^{12}$  cells with a varying composition and community.<sup>4</sup> The most common microbes present in the gut are Bacteroidetes, Bifidobacterium, Clostridium, Lactobacillus and Enterococcus.<sup>5</sup> Studies shows obesity is one of the major health problems associated with the intestinal microbiota.<sup>6</sup> In developing countries, more than 500 million people are obese, which leads to various health problems and consequences that includes diabetes mellitus, cancer, cardiovascular diseases, non-alcoholic fatty liver etc.<sup>7</sup> The composition of the diversity in bacterial species varies between normal & obese, increasing the number of Firmicutes to damage Bacteroidetes in obese patients.<sup>8</sup>

Microbiota varies to every individual. Influence of gut microbes in healthy and diseased human have been reported in research studies. Finally, the proof that links gut bacteria to the metabolism of host has shown the methods to treat obesity through therapeutic methods.<sup>7,9,10</sup> Modifying the composition of gut microbiome in human help revealing the solution to study the function of gut microbes in obesity individual.<sup>11,12</sup> Consumption of high level of simple sugars and saturated fat could be contributed to obesity, by altering the composition and function of the microorganisms that colonize in the gastro intestinal tract. The accurate pathways by how the gut microbiota contribute to obesity, remains largely unknown.<sup>9,13</sup> By increasing systemic inflammation and affecting vagal nerve activity, gut microbiota indirectly influences the physiological gene expression and promote overeating. The functions of the gut microbiota are being investigated to produce novel therapeutic strategies against diet induced obesity.<sup>14</sup> Gut microbiota impacts in the processing of insoluble dietary fiber in the colon region.<sup>15-17</sup>

Probiotics are currently recognized for several health benefits and they are used as a complementary therapeutic agent for treating many metabolic disorders. Probiotics has the ability to alter the intestinal microbiota, altering the energy metabolism and lipid metabolism.<sup>18</sup> Short chain fatty acids (SCFA's) produced by probiotic microbes, present in the gastro intestinal tract has certain mechanisms in the host.<sup>19-22</sup> Probiotics can also reduce the cardio vascular diseases by reducing the high cholesterol level.<sup>23-25</sup> Insoluble dietary fiber sources may create an impact in their processing by the gut microbiota present in the colon<sup>26</sup> short chain fatty acid production, especially butyrate has lot of health benefits but it is limited in the body by the lack of dietary fiber.<sup>4,11,27,28</sup>

Patricia<sup>29</sup> reported that obesity is a multifactorial disease and it is a risk factor for several non-communicable diseases such as heart attacks, type 2 diabetes mellitus etc. Gut microbiota and the dietary intake of an individual, especially

lipids have high potential as the modulatory fact of the gut microbiota composition. Lipids act as a key factor and it links microbiota with obesity. Gut microbes interacts with the host metabolism and develops obesity through fatty acids. Both saturated and poly unsaturated fatty acids create the impact in host and the gut microflora. The study concludes the endocannabinoid system present in the human body is controlled by gut microbiota and the imbalance in this maintenance results in the development of obesity.<sup>30-32</sup>

One of the studies discussed mainly about the gut microbiota associated atherosclerosis and hypertension and it also reveals the prospectus of gut microbiota related therapeutic strategies for the treatment of coronary diseases for the human in the future.<sup>33</sup> Gut dysbiosis is associated with the pathogenesis of many types of diseases which includes obesity, cancer, type II diabetes mellitus, fatty liver and certain heart diseases.<sup>11,34</sup>

Recent researches on the gut microbiota advances the therapeutic usage of those microbes and the application of their metabolites after fermentation in human body. However, their exact metabolic pathways are still unknown. The study reported on the involvement of gut microbes and their functional components in improvement of atherosclerosis. Treatment with potential probiotics will help in preventing atherosclerosis.<sup>1,8,29,35,36</sup>

The gut microbiota acts as a critical factor which influences the metabolism of cholesterol. the impact and influence of the gut microbiota in the metabolism of cholesterol and its association with metabolic dysfunctions and the potential of altering and modulating the gut microbiota as a challenging therapeutic product to reduce the level of cholesterol.<sup>18,37-40</sup>

## 2. Materials and Methods

### 2.1. Sample collection

Human faecal samples were collected from selected healthy individuals. The study was approved by the Ethical committee of St. Joseph's College of Arts and Science (Autonomous), Cuddalore, Tamil Nadu for collecting faeces samples. The samples were collected in sterile containers, transported to the laboratory immediately and subjected to microbiological analysis.

### 2.2. Microbial and biochemical analysis of the isolates

Human faecal samples were collected in a sterile container. Freshly collected faecal sample was directly inoculated into MRS, TSB, MacConkey and Lactobacillus oxgall agar. The plates were incubated aerobically and anaerobically at 37°C for 24-48 hours. The isolates were tested for their Morphological, Motility, Catalase, Oxidase tests and Biochemical tests.

### 2.3. Lecithinase producing ability of the isolates

Lecithinase test was performed to determine the potential of the microbes to produce the Lecithinase enzyme by method with slight modification.<sup>41</sup> The test was performed using Tryptic soy agar emulsified with egg yolk. Egg yolk contains large amount of Lecithovitellin. Microbes which are able to produce Lecithinase enzyme will use the lecithin, present in the egg yolk as a substrate. The 24 hours inoculated active cultures are patch streaked in the media and incubated at 37°C for 3-4 days, anaerobically. If the bacteria produce the enzyme, there will be appearance of white opaque zone around the inoculum.

### 2.4. Cholesterol degrading ability of the isolates

Microbes which are able to produce the enzyme cholesterol oxidases can degrade cholesterol and convert them into 4-cholesten-3-one. Cholesterol (5- Cholesten-3beta-ol) is purchased from Hi-media. Cholesterol degradation test was performed in the minimal salt agar, by adding cholesterol as a sole carbon source. About 200 milligrams of Cholesterol was first dissolved in 1ml of chloroform, adjusted to pH 7 and transferred into the media aseptically.<sup>24</sup> The 24 hours inoculated active cultures were patch streaked in the media and incubated at 37°C for 5-6 days. If the bacteria produce the enzyme cholesterol oxidases, clear halo zone around the streak was formed if the bacteria produce the enzyme cholesterol oxidases.

### 2.5. Gas chromatography analysis of short chain fatty acids

#### 2.5.1. Sample Preparation

480µl of periodic acid (100 mM) was taken and added to 300µl of formic acid (10%) in a screw cap tube, containing 720µl of sample and the tube was shaken vigorously. The mixture was then closed tightly and placed in the boiling water bath at 100°C for 60 min. The screw cap tube was cooled at room temperature for 15 min after treatment with water bath and then kept in the refrigerator for 20 min. After cooling, the mixture in the closed screw cap tube was mixed using a vortex mixer for 30 seconds. The mixture was then transferred to a 1.5 ml Gas Chromatography vial and then it was ready for injection into the GC. All the injections loaded were 0.4µl in volume and performed with a 10-µl syringe autosampler. The syringe was washed thrice subsequently with methanol (98%) and double distilled water for three times, before and after each injection of the samples.

#### 2.5.2. Analysis

A Shimadzu GC-2010 Plus gas chromatograph, equipped with a straight deactivated direct injector liner with length of 2 mm and a Alltech EC-5 column of length 15m with flame-ionization detector was utilized. The carrier gas, used was nitrogen gas with a flow rate of 1.2 ml/min, and at

the inlet sample was split 100:1. The injection volume was set at 0.4µl. The run time was programmed at 35 min. The initial temperature for oven was 50°C–250°C. The injector and detector temperatures were set at 250 and 300°C, respectively. Hydrogen and air flow rates at the FID were set at 30 and 400 ml/min, respectively. The peak area output signal was computed via integration using the GC V.2.0 software.

### 2.6. Molecular Identification of the Isolate

#### 2.6.1. DNA Extraction

Bacterial cells were cultured overnight and separated by centrifugation at 13,000-16000rpm for 2 min at room temperature. Template DNA was extracted from cell pellet using GenElute Bacterial Genomic DNA kit (Sigma-Aldrich, Mumbai). The culture medium was removed and the cell pellets were re-suspended in 200µl of lysozyme solution and incubated at 37°C for 30 minutes. 20µl of RNase A solution was added and incubated for 2 minutes at room temperature, after incubation. 20µl of Proteinase K solution was added and incubated for 10 min at 55°C. 200µl of lysis solution C was added to the cell suspension vortexed thoroughly and incubated at 55°C for 10 mins. 200µl of ethanol was added to the lysate and mixed thoroughly. The lysate was transferred onto column and centrifuged at 10,000 rpm for 1 min and the flow through was discarded. The column was placed on the same 2ml collection tube. 500 µl of Wash solution was added to the column and centrifuged at 10,000 rpm for 1min. The flow through was discarded and the collection tube was reused. 500µl of wash solution 1 was added to the column and centrifuged for 3min at 13,000- 16,000 rpm. The column was transferred to a new collection tube, 200µl of the elution buffer was added directly onto column without spilling and incubated.

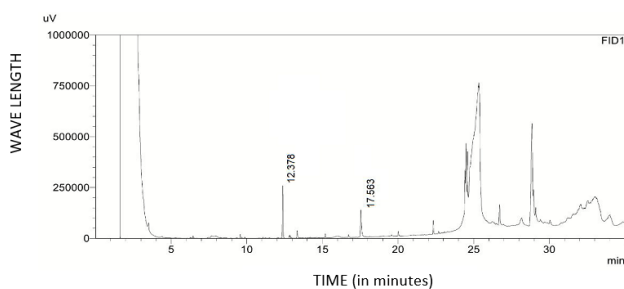
#### 2.6.2. PCR Amplification

The isolates were further confirmed by amplifying and sequencing the 16S rRNA which are of the length of 1400 base pairs. Amplification was performed using the universal primer 27-F and 1492-R. PCR amplification was performed in 25 µl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 20 pmol of each primer, a 0.2 mM concentration each of the four de-oxynucleotide triphosphates, 0.5 U of Taq DNA polymerase (Sigma-Aldrich, USA), and template DNA (10 ng). Amplifications were carried out for 34 cycles (94 °C for 1 min, 56 °C for 30 s and 72 °C for 1 min) in Eppendorf PCR with an initial denaturation of 94 °C for 4 min and a final extension of 72 °C for 8 min. Amplicons were detected by electrophoresis on a 1.5% agarose gel staining with ethidium bromide. The sequences were analyzed with the BLAST (NCBI) for the identification of bacteria.<sup>42,43</sup>

### 3. Result

Thirty isolates were collected from the stool of the selected healthy subjects for this investigation. Seventeen of these isolates were found to be gram positive bacteria, whereas the remaining isolates were found to be gram negative. Every isolate displayed negative results for both catalase and oxidase. All isolates were urease negative, with the exception of two (MB201 & MB221). With another exception of two isolates (MB216 & MB217), all of the isolates were found to be non-motile; these findings are significant because they are in accordance with the Bergey's manual of classification. In the current study, all the bacteria isolated from gut microbiota exhibited negative result for the toxic enzyme Lecithinase production. This indicates that the isolates were non-toxic to the host system and can be used to develop probiotic microorganisms. Gut microbiota has high potential to degrade the cholesterol. The ability them to produce short chain fatty acids, which helps in the fat burning metabolism. Among the seventeen isolates, only two isolates were found to be positive. Isolates MB224 and MB565 produced halo zone around the inoculums. Out of 17 isolates, only two isolates were chosen based on other properties for analysis of lactic acid and butyric acid by GC-FID.<sup>44</sup> Both isolates were gram positive, urease negative and non-motile. Isolate MB224 produces 16.492 PPM of lactic acid and 12.362 PPM of butyric acid. Isolate MB218 produces 8.348 ppm of lactic acid and 9.817 ppm of butyric acid (Figures 1 and 2).

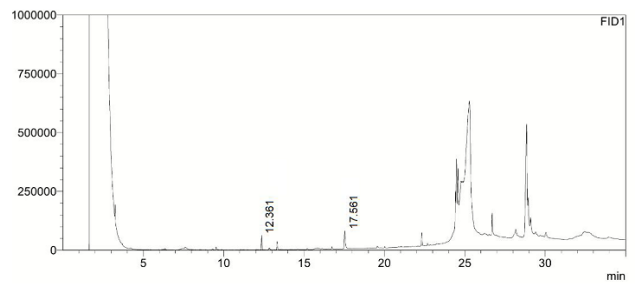
Based on the results of the two isolates, which exhibit good functional properties, a molecular methodology employing 16srRNA sequencing was employed to identify both. It was determined that the isolates were *Enterococcus faecium* MN294515 and *Enterococcus faecium* MN535170.



**Figure 1:** Short chain fatty acid analysis of the isolate MB224 by Gas chromatography

### 4. Discussion

The current study narrates about the gut microbiome and their vital characteristics in the development of probiotics. Many research studies were been submitted related to the cholesterol degradation by the gut bacteria, which in turn



**Figure 2:** Short chain fatty acid analysis of the isolate MB218 by Gas chromatography

**Table 1:** Molecular Identification of the Selected Isolates

S.No.	Culture id	Organisms identified	Genbank accession number
1	MB218	<i>Enterococcus faecium</i>	MN294515
2	MB224	<i>Enterococcus faecium</i>	MN535170

helps to treat obesity in human.<sup>7,45</sup> The production of short chain fatty acids observed to be the major characteristics of gut bacteria. Short Chain Fatty Acids are involved in the treatment of irritable bowel syndrome and it is also used in the treatment of many diseases such as cancer and diabetes. It also increases the metabolism of fat and cholesterol.<sup>46</sup> Lactic acid and butyric acid are involved in the regulation of the immune system. It also mediates the epithelial cells, lining on the gut. It has the ability to treat irritable and inflammatory bowel diseases.<sup>6,17,47</sup> At the cellular level, these short chain fatty acids are involved either directly or indirectly to create a good effect in cell proliferation, cell differentiation and gene expression. Similar studies have shown that the bacterial strains *Bacillus pumilus* W1 and *Serratia marcescens* W8 produce the enzyme cholesterol oxidase extracellularly and have the capacity to degrade cholesterol.<sup>22,41,46,48</sup> Accumulation of cholesterol inside the tissues makes them to lose its viability and function and it also leads to many neurodegenerative diseases, atherosclerosis and other liver problems.<sup>8</sup> Another study reported that out of 567 lactic acid bacterial strains that were isolated from human faeces samples, 36 of them carried probiotic properties.<sup>20</sup> The presence of lactic acid bacteria in the stool samples from dyspepsia patients as well as healthy individuals.<sup>23</sup> Cholesterol oxidase is an enzyme, which is produced by certain kind of bacteria, which converts cholesterol into coprostanol. This enzyme involves in the catalysis of cholesterol and they also act as co factors for FAD.<sup>10</sup> Gram negative bacteria present in the gut mostly produce acetate and propionate. Gram positive bacteria mainly produce butyrate and lactate.<sup>27</sup>

One of the important toxic enzymes, produced by bacteria is lecithinase, which has phospholipolytic activity and involved in the hydrolysis of lecithin. They were also termed as Phospholipase C and it usually acts on the cell membrane of the animal tissues, which contains lecithin.<sup>49</sup> The toxicity of the lecithinase is mainly due to the enzymatic action of lecithinase onto the cell membranes, which leads to the cell lysis in the host or by breaking the phospholipids present in the cell membrane.<sup>50</sup> In the current study, all the bacteria isolated from gut microbiota exhibited negative result for lecithinase production. Also, the selected two isolates *Enterococcus faecium* MN294515 and *Enterococcus faecium* MN535170 in this study showed a very good property in short chain fatty acid production and also does not produce lecithinase enzyme. This indicates that the isolates were non-toxic to the host system and can be used to develop probiotic microorganisms. By offering probiotic support, this study can be further investigated for the treatment of obesity in humans.

## 5. Conclusion

Gut bacteria are essential because they help with physiology and even psychology by addressing a wide range of conditions like diabetes, high cholesterol, stress, and other conditions. Thus, this study clarifies how crucial it is to preserve the gut microbiota in order to tolerate and stabilize the body's enzymatic and hormonal functions. The two isolates *Enterococcus faecium* MN294515 and *Enterococcus faecium* MN535170 that were found can be further investigated in order to create and develop the finest probiotic for treating obesity, a condition that has long been popular.

## 6. Source of Funding

None.

## 7. Conflict of Interest

The authors declare no conflict of interest.

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