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Gut microbiota is not modified by Randomized, Double-blind, Placebo-controlled Trial of VSL#3 in Diarrhea-predominant Irritable Bowel Syndrome

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Abstract

Irritable Bowel Syndrome (IBS) is a common condition that negatively impacts the quality of life for many individuals. The exact etiology of this disorder is largely unknown; however, emerging studies suggest that the gut microbiota is a contributing factor. Several clinical trials show that probiotics, such as VSL#3, can have a favorable effect on IBS. This double-blind, randomized placebo-controlled study has been conducted in diarrhea-predominant IBS subjects in order to investigate the effect of VSL#3 on the fecal microbiota. The bacterial composition of the fecal microbiota was investigated using high-throughput microarray technology to detect 16S RNA. Twenty four subjects were randomized to receive VSL#3 or placebo for 8 weeks. IBS symptoms were monitored using GSRS and quality of life questionnaires. A favorable change in Satiety subscale was noted in the VSL #3 groups. However, the consumption of the probiotic did not change the gut microbiota. There were no adverse events or any safety concerns encountered during this study. To summarize, the use of VSL#3 in this pilot study was safe and showed improvement in specific GSRS-IBS scores in diarrhea-predominant IBS subjects. The gut microbiota was not affected by VSL#3 consumption suggesting that the mechanism of action is not directly linked to the microbiota.

Keywords

Irritable Bowel Syndrome; Diarrhea; Probiotics; Microbiota

Introduction

Irritable Bowel Syndrome (IBS) is the most common disorder encountered by gastroenterologists. It can be disabling with significant impact on the quality of life, yet the exact etiology and pathophysiology remain undefined. Many theories suggest immune activation with low-grade intestinal inflammation and an altered host brain-gut response. Recently, the gut microbiota is being considered an important contributor to the development of IBS; based on observations of post-infectious symptoms, response to probiotics or specific antimicrobials, the relapse of symptoms with the use of antibiotics that change the microbiota, and finally, the suspicion of small bowel bacterial overgrowth. It is estimated that the human colon contains up to 10^{14} bacteria [1]. Although this diverse microbial load is largely beneficial, it has been postulated that altered bacterial populations or products of bacterial metabolism may contribute to human disease. The mechanisms by which altered fecal microbiota induce disease are poorly understood. Fecal short chain fatty acids produced by microbiota are critical for colonic epithelial maintenance, yet they are

clearly different in IBS [2]. Maximal gas production and hydrogen excretion after lactulose is increased in IBS as colonic-gas production, particularly of hydrogen, is greater in patients with IBS than in controls, and both symptoms and gas production are reduced by an exclusion diet. This reduction may be associated with alterations in the activity of hydrogen-consuming bacteria further emphasizing the importance of fermentation in the pathogenesis of IBS [3]. Moreover, increased bacterial methane production has been linked to constipation in IBS [4]. In this population, serotonin release is blunted, suggesting a possible neurochemical basis for impaired motor function[5]. Now, several studies have demonstrated that patients with IBS have altered gut microbiota[6-9] leading to the development of probiotic studies that suggest a favorable effect on IBS[10-12]. One of the questions that remain unanswered is how the probiotic products influence the gut microbiota. VSL#3 is a polymicrobial probiotic product that shows promise in treating IBS[13-15]. This pilot study was designed to investigate the safety, efficacy and the effect of VSL#3 on the gut microbiota of patients with diarrhea-predominant IBS.

Methods

Clinical Study

Protocol and assignment—Twenty four subjects with diarrhea-predominant irritable bowel syndrome that met the Rome III criteria were randomly and blindly assigned to consume VSL#3, 900 billion bacteria per day or placebo composed of cornstarch (base ingredient for probiotic product) for eight weeks. Subjects were excluded if they have been on probiotics or antibiotics for three months prior to enrollment. The safety of the product was monitored closely throughout the study. IBS symptoms were monitored using GSRS; a clinical rating scale for gastrointestinal symptoms in patients with irritable bowel syndrome on a weekly basis. Quality of life (QOL) was monitored using QOL questionnaires at baseline and week 8. Fecal samples were collected at baseline, week 4 and week 8. The primary goal of this study was to examine the effect of the probiotic VSL#3 on the gut microbiota in diarrhea-predominant. This study was approved by the Institutional Review Board. Subjects were electronically randomized through the pharmacy department. Compliance was monitored by asking subjects to bring all used and unused study medication sachets.

Masking—The group assignment of subjects was concealed to the subject and all the investigating team.

Microbiota study

The bacterial make up of the gut microbiota was investigated using high-throughput microarray technology as we previously reported[16]. All fecal specimens were homogenized and immediately frozen at -80°C .

DNA isolation

Fecal DNA was isolated using ZR Fecal DNA kit (Zymo Research Corporation, Orange, CA). Frozen fecal samples (150 mg) were used following the manufacturer's protocol. Eluted DNA was checked for quality by 1% agarose gel electrophoresis. The eluted DNA was then quantified on a NanoVue Spectrophotometer (GE Health care, NJ).

PCR reaction

The genomic DNA (gDNA) was diluted to a concentration of 400 ng/ μl and universal primers "16S rRNA for (forward AGA GTT TGA TCC TGG CTC AG)" and "16S rRNA

Rev (reverse ACG GCT ACC TTG TTA CGA CTT)” from Integrated DNA technologies, were used to amplify the whole 1.5kb fragment of 16S ribosomal RNA gene.

The reaction was carried out for 30 cycles on StepOne Real-time PCR system (Applied Biosystems, Foster City, CA) as a normal thermo-cycling reaction, for 90 seconds at 75°C. The amplified product was cleaned up using Qiagen MinElute PCR Purification Kit as per the manufacturer’s suggested protocol. The amplified product was verified by 1% agarose electrophoresis alongside 100 bp Low Scale DNA Ladder (Fisher Scientific, Pittsburgh, PA) for the presence of a distinct band at 1500 bp position. The amplified DNA was then quantified on a NanoVue Spectrophotometer.

DNA fragmentation

Amplified 16S rDNA (400 ng) was used as target for the microarrays. DNase I enzyme (New England Biolabs, Ipswich, MA) was diluted to a concentration of 0.05 units/μl in DNase reaction buffer. Ribosomal DNA fragmentation was carried out using working concentration of enzyme at 0.05 units DNase I/ μg of amplified DNA as this concentration has been shown to produce ideal fragment size for microarray hybridization¹⁶. The DNA was incubated at 37°C along with the enzyme for 10 minutes; thereafter the enzyme was heat-inactivated at 75°C for another 10 minutes.

DNA labeling reaction

The single color protocol from Agilent Technologies (version 5.0, June 2007) was adopted for DNA labeling reaction using Agilent Genomic DNA Enzymatic Labeling Kit (Agilent Technologies, Santa Clara, CA) and using single dye (either with Cy-3 or Cy-5). The thermo-cycler was programmed for the following temperatures: 95°C, 37°C and 65°C. To 13 μl of the fragmented DNA, 2.5 μl of Agilent primers were added and the mixture was incubated at 95°C for 3 minutes and then cooled to 4°C. The samples were briefly spun in a microcentrifuge to collect the contents at the bottom. Reaction buffer, dNTPs, dye (Cy-3 or Cy-5) and Exo-Klenow fragment was added to the annealed primer-template mix and incubated at 37°C for 2 hours followed by heat inactivation with the enzyme at 65°C for 10 minutes. The labeled DNA was mixed of 1X TE buffer (pH 8.0) and passed through Microcon YM-30 filters to clean the DNA. There were 38,340 probes on the array with more probes than signals obtained.

Microarray hybridization

The following components were added in the order indicated: Cy-3 or Cy-5 labeled DNA, Cot-1 DNA, Agilent 10X blocking agent, and Agilent hybridization buffer. Components were mixed thoroughly, quickly spun and then incubated at 95°C for 3 minutes and 37°C for 30 minutes. The reaction mixture (40 μl) was loaded onto a gasket chamber and the printed microarray was placed over the gasket with the active side of the array facing down. The sandwich assembly was thoroughly inspected for any leaks then hybridized for 24 hours. Washing and scanning (Agilent G2565CA Microarray scanner with SureScan High resolution technology, Agilent Technologies, Santa Clara, CA) were then carried out as per manufacturer’s protocol and the raw data was extracted using Agilent Feature Extraction software (Version 10.1).

Statistical Analyses

Microarray Data—Signals from 852 separate bacteria were obtained from the 15 VSL patients at baseline, week 4, and week 8. The bacteria were classified into 120 different genera, 53 families, 25 orders, 14 classes, and 8 phyla. The signal from each bacterium for each patient was then divided by its genus average copy number to obtain an adjusted signal

for each bacterium. The 852 adjusted signals were summed and the percent of the total adjusted signal was determined for all bacteria. Adjusted signals were then summed by genus, family, order, class, and phylum and the percents of the total signal were determined for each hierarchical classification level.

For statistical analyses, all adjusted signals were transformed using the natural logarithmic transformation. Comparisons between the baseline, week 4 and week 8 signals were made with repeated measures analysis of variance. P values were adjusted for multiple comparisons using Bonferroni corrections.

Results

Clinical Outcomes

Twenty four subjects were enrolled in this study; 15 were randomly assigned to the VSL #3 group and 9 to the placebo group. Two thirds of the patients in each group were females, twenty two patients were white, and two were African-American. The mean ($\pm$ SD) age was 21.8 ± 17 years, and mean weight was 80 ± 27 kg (range 52-132 kg). There were no differences between the groups for age and weight, and both groups' weights remained stable throughout the treatment period.

GSRS-IBS pain subscale scores decreased significantly over the treatment period in both groups, but there was no difference between the groups in the degree of change (Table 1). The mean baseline pain score for both groups combined was 3.5 ± 1.2 , and decreased by 1.6 ± 1.3 points at the end of the treatment period. Significant decreases also occurred in the combined groups in the bloating, diarrhea, satiety, and global scores. The decrease was significantly greater in the VSL #3 group for the satiety subscale.

Significant decreases in scores were also observed in the combined groups for 3 of 7 QOL statements and the overall QOL score, with no differences between the groups (Table 2). The baseline QOL score was 2.7 ± 1.1 and decreased to 1.9 ± 0.7 after 8 weeks. The patients reported that they missed from 0-4 activities and 0-2 work days, and experienced from 0-2 significant life events during the 8 weeks prior to enrollment. There were no differences between the groups, and no changes occurred during the 8-week treatment period.

Safety

There were no adverse events noted during the study.

Microarray Data

Of the 852 phylotypes analyzed, no differences were noted after adjustment for multiple comparisons. The number of species and percents of total signals by phylum and class are shown in Table 3. The class Clostridia contained the largest number of species (564) and comprised over 60% of the total signals at baseline, week 4, and week 8. There were no differences noted at baseline or with probiotic consumption at the Phylum, Class, Order, Family or Genus levels. Similarly, there was no change in the gut microbiota after placebo consumption (table 4).

Discussion

This study is the first placebo-controlled trial reporting culture-independent analysis of the gut microbiota after consumption of VSL#3. Abnormalities in intestinal microbiota have recently surfaced as a contributing factor to the development of irritable bowel syndrome. A significant reduction in *bifidobacteria* of the gut microbiota can result in an increased

production of gas, dysmotility, and enteric neuromuscular dysfunction[17] which in turn can contribute to the development of IBS. Since gut microbiota in IBS is different from that of healthy subjects[18-20], probiotic-related changes in the enteric flora could reduce the non-desirable effects of bacteria in the gut[3] and favorably influence gut function. Probiotics, defined as live or attenuated bacteria or bacterial products that confer a significant health benefit to the host[21] have the potential to serve as a tool to explore gut microbial interactions. Probiotics, especially VSL#3 have therapeutic potential as they possess antibacterial and antiviral effects that could potentially prevent or ameliorate post-infectious IBS[22, 23]. They also have mucosal anti-inflammatory properties[24, 25] with an increase in mucus[26] production and reduction of the migration of neutrophils to the intestinal epithelium[27]. Reduction of mucosal inflammation can ameliorate the consequences of inflammation and reduce the neurochemical and impaired motor function found in many subjects with IBS. Furthermore, probiotics could alter stool and gas formation[28-30] or increase intestinal mucus secretion[31, 32], which could ameliorate symptoms such as constipation and diarrhea. Indeed, many probiotic studies suggest a favorable outcome in ameliorating symptoms of irritable bowel syndrome[10, 33, 34]. While results between studies are difficult to compare because of differences in study design, probiotic dose, and strain, there has been evidence of symptom improvement[14, 15, 35-38]. Several of these studies have involved either *lactobacilli* or *bifidobacteria*[39]. One of the probiotic products studied with success in this disorder is VSL#3[14, 15], a product that combines both bacterial groups. However, many of the studies are limited by lack of documentation of stool recovery of the probiotic agent and lack of knowledge of how the probiotic agent can modify the gut microbiota.

Whether probiotics exert their effect on the host through alteration of the gut microbiota remains a controversial issue. Animal studies confirm the ability of VSL#3 to modify the microbiota. VSL#3 strains survived in the gastrointestinal tract, increased the cecal concentrations of *bifidobacteria*, and modified cecal microflora metabolic activity in mice [40]. In human subjects with irritable bowel syndrome, the data is scarce. Only one study could be identified in the literature that has addressed this issue. Brigidi et al[13] investigated the gut microbiota in 10 human subjects with irritable bowel syndrome and functional diarrhea but used culture methodology. Most of the intestinal microbiota species are obligate anaerobes [41-43] making it difficult and inaccurate to depend on culture techniques to investigate the microbiota. Nevertheless, Brigidi could not find any significant changes in the gut microbiota as a result of VSL#3 use. Similarly in our study, the fecal gut microbiota did not appear to be affected by VSL#3 suggesting that alteration of the gut microbiota is not the mechanism by which this probiotic product can offer benefit to the host relevant to the findings in this study showing a beneficial effect on the “Satiety” parameter.

Recent studies suggest that obesity is associated with a change in the gut microbiota[44]. Therefore, analysis of the BMI of subjects was performed to determine if there was a difference between the two groups. We found no statistical difference in the BMI of subjects receiving placebo versus those receiving the probiotic product. Therefore, changes based on BMI would not be a contributing variable in this study.

This study is the first to describe a culture-independent molecular approach to the interrogation of the gut microbiota in human subjects with diarrhea-predominant IBS consuming VSL#3.

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Table 1

GSRs-IBS scores and comparisons between the VSL #3 and placebo groups

Subscale	Group	Baseline	W1	W2	W3	W4	W5	W6	W7	W8	P Values ¹
Pain	VSL #3	4.0±1.3	1.9±0.7	2.7±1.2	1.7±0.8	2.4±0.7	2.6±1.0	2.1±1.0	2.0±0.6	1.9±0.9	.81, <.001, .28
	Placebo	3.0±1.0	2.0±1.2	2.6±1.3	2.4±1.1	2.0±1.0	2.2±1.0	1.9±0.7	2.1±1.0	2.0±0.9	
Bloating	VSL #3	2.7±0.9	1.1±0.2	2.1±0.9	1.9±0.6	1.7±0.3	1.9±0.2	1.5±0.3	1.9±1.0	1.6±0.3	.52, .01, .15
	Placebo	1.6±0.4	1.3±0.5	1.7±0.7	1.7±1.1	1.5±0.8	1.7±1.0	1.6±1.2	1.5±0.9	1.5±0.9	
Constipation	VSL #3	1.8±0.8	1.5±0.5	1.4±0.5	2.0±1.0	1.5±0.5	1.1±0.2	1.4±0.5	1.5±0.9	1.2±0.4	.58, .32, .42
	Placebo	2.2±1.2	1.2±0.4	2.3±2.2	1.4±0.9	1.8±0.6	1.5±0.5	1.9±1.2	1.5±0.7	1.5±0.7	
Diarrhea	VSL #3	3.1±1.6	1.6±0.5	2.6±1.3	1.9±0.8	1.7±0.7	2.2±1.2	1.5±0.4	1.5±0.5	1.6±0.5	.56, <.001, .89
	Placebo	3.1±1.2	2.5±1.3	2.6±1.5	2.4±1.5	2.1±0.9	2.3±0.9	1.9±0.9	1.9±0.9	1.8±0.8	
Satiety	VSL #3	2.3±0.9	1.2±0.3	1.8±1.2	1.2±0.4	1.3±0.4	1.1±0.2	1.1±0.2	1.1±0.2	1.0±0.0	.65, .01, .02
	Placebo	1.8±1.3	1.6±0.9	1.4±0.9	1.7±1.3	1.7±1.6	1.5±1.1	1.8±1.8	1.5±1.1	1.5±1.1	
Global score	VSL #3	2.8±0.7	1.5±0.2	2.2±0.9	1.8±0.3	1.7±0.2	1.8±0.4	1.5±0.2	1.6±0.3	1.5±0.3	.81, .001, .51
	Placebo	2.4±0.6	1.8±0.7	2.2±1.1	2.0±1.2	1.8±0.8	1.9±0.7	1.8±1.0	1.7±0.8	1.7±0.8	

Values in table are the mean±SD. W1-W8 are treatment weeks 1-8.

¹ P values are the group, treatment, and interaction p values respectively from two-way ANOVAs.

Table 2
Quality of life (QOL) scores and comparisons between the VSL #3 and placebo groups

QOL statement	VSL #3		Placebo		P Values [/]
	Baseline	W8	Baseline	W8	
I am bothered by how much time I spend on the toilet	2.8±1.8	2.0±0.7	2.5±0.6	2.5±1.7	.90, .44, .44
I feel I get less done because of my bowel problems	3.0±1.2	1.6±0.5	2.0±0.8	1.5±0.6	.22, .05, .30
I avoid stressful situations because of my bowel problems	3.2±1.3	2.0±0.7	2.3±1.5	1.8±1.0	.41, .05, .36
I watch the kind of food I eat because of my bowel problems	4.2±1.3	2.8±1.3	3.3±1.5	2.0±0.0	.25, .01, .86
It is important to be near a toilet because of my bowel problems	3.0±1.4	2.0±1.2	2.0±1.2	1.5±0.6	.31, .09, .53
I worry about losing control of my bowels	2.2±1.8	1.6±0.9	2.5±1.7	1.5±0.6	.89, .19, .73
Overall average score	3.0±1.3	2.1±0.8	2.4±1.0	1.8±0.6	.41, .05, .63

Values in table are the mean±SD. W8 is treatment week 8.

[/] P values are the group, treatment, and interaction p values respectively from two-way ANOVAs.

Table 3

Microarray analysis data for the VSL #3 subjects

Phylum	Class (Subclass)	No. of species	Baseline	W4 (p-value)	W8 (p-value)
Actinobacteria	Actinobacteria (Actinobacteridae)	20	2.1±0.5	1.6±0.2	1.6±0.3
	Actinobacteria (Coriobacteridae)	9	2.6±0.6	2.3±0.3	2.4±0.3
Bacteroidetes	Bacteroidia	142	16.2±6.1	19.6±2.4	19.7±1.9
Firmicutes	Bacilli	20	3.7±2.7	3.1±0.9	3.1±0.4
	Clostridia	564	62.7±5.3	62.4±2.9	62.6±2.5
Fusobacteria	Erysipelotrichi	39	5.4±2	5.0±0.4	4.8±0.5
	Fusobacteria	3	0.3±0.1	0.3±0.2	0.3±0.1
Lentisphaerae	Lentisphaeria	1	0.5±0.2	0.4±0.1	0.3±0.1
Proteobacteria	Alphaproteobacteria	9	0.6±0.2	0.6±0.2	0.5±0.1
	Betaproteobacteria	18	1.9±1.7	1.2±0.5	1.2±0.7
	Deltaproteobacteria	5	0.8±0.4	0.7±0.2	0.7±0.1
	Epsilonproteobacteria	6	0.9±0.3	0.9±0.0	0.9±0.1
Spirochaetes	Gammaproteobacteria	11	1.3±0.8	1.0±0.1	0.9±0.1
	Spirochaetes	4	0.8±0.2	0.8±0.1	0.8±0.0
Verrucomicrobia	Verrucomicrobiae	1	0.2±0.1	0.1±0.1	0.1±0.1

Values in table are the percents of the total adjusted signals (mean±SD). W4 and W8 are treatment weeks 4 and 8. None of the comparisons had a statistically significant p-value (adjusted p-value<0.05).

Table 4

Microarray analysis data for the placebo subjects

Phylum	Class (Subclass)	No. of species	Baseline	W4 (p)	W8 (p)
Actinobacteria	Actinobacteria (Actinobacteridae)	20	1.1±0.1	1.2±0.1 (0.91)	1.3±0.1 (0.43)
	Actinobacteria (Coriobacteridae)	9	1.0±0.07	1.1±0.09 (0.91)	1.1±0.09 (0.61)
Bacteroidetes	Bacteroidetes	142	14.9±1.4	14.9±1.03 (0.91)	16.4±2.3 (0.43)
Firmicutes	Bacilli	20	1.9±0.4	1.7±0.5 (0.91)	1.8±0.5 (0.43)
	Clostridia	564	75.7±2.3	75.8±1.4 (0.91)	73.9±2.7 (0.43)
	Erysipelotrichi	39	2.3±0.3	2.5±0.4 (0.94)	2.6±0.3 (0.43)
Fusobacteria	Fusobacteria	3	0.1±0.05	0.12±0.01 (0.96)	0.12±0.03 (0.43)
Lentisphaerae	Lentisphaerae	1	0.08±0.02	0.07±0.01 (0.91)	0.06±0.02 (0.68)
Proteobacteria	Alphaproteobacteria	9	0.1±0.04	0.17±0.02 (0.91)	0.2±0.03 (0.43)
	Betaproteobacteria	18	0.8±0.2	0.6±0.1 (0.75)	0.6±0.2 (0.43)
	Deltaproteobacteria	5	0.2±0.04	0.2±0.0 (0.91)	0.29±0.02 (0.96)
	Epsilonproteobacteria	6	0.2±0.02	0.2±0.01 (0.91)	0.3±0.03 (0.43)
	Gammaproteobacteria	11	0.7±0.1	0.7±0.08 (0.91)	0.8±0.1 (0.43)
Spirochaetes	Spirochaetes	4	0.1±0.02	0.1±0.01 (0.91)	0.1±0.1 (0.43)
Verrucomicrobia	Verrucomicrobiae	1	0.07±0.05	0.07±0.05 (0.91)	0.05±0.05 (0.65)

Values in table are the percents of the total adjusted signals (mean±SD). W4 and W8 are treatment weeks 4 and 8. None of the comparisons had a statistically significant p-value (adjusted p-value<0.05).