EVALUATION OF MICROBIAL DIVERSITY PRESENT IN HERBAL SUPPLEMENTS AS REVEALED BY PCR-BASED 16S RRNA SEQUENCE ANALYSIS

by
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ABSTRACT

Stephen Stone: Evaluation of microbial diversity present in herbal supplements as revealed by PCR-based 16S rRNA sequence analysis

Over the last few decades people have become more aware of their general wellness and have turned towards alternative measures to ensure good health. One of these alternative measures, the herbal supplement market, has risen significantly in recent years, even though there is no conclusive research that points to the effectiveness of herbal supplements. Also, because of sparse regulation from the FDA, there are many questions related to the efficacy, composition, processing methods, and, consequently, safety of these supplements. The aim of this study was to determine the microbial composition of herbal supplements in an attempt to identify potential targets for both effectiveness and dangers. Five out of six herbal supplements tested contained evidence of bacterial DNA, with Gingko Biloba being the only exception. Dominant bacterial species or groups detected in multiple samples were *Salmonella enterica, Lactobacillus spp, Shigella sonnei, Salmonella paratyphi a, Escherichia_Shigella spp,* and *Clostridium spp.,* although 27 different species or species groups were identified. *S. enterica* and *Lactobacillus spp.* were the most proportionally abundant species in most samples, representing a potential pathogen (*S. enterica*) and a potentially beneficial bacterium (*Lactobacillus spp.*), although neither of these species, or the others detected, could explain the variability in efficacy of supplements. The presence of DNA from potential pathogens in herbal supplements, along with that from bacterial cells that should only present in humans or animals, suggests that further regulation and/or moderation is needed so that herbal supplements will be adequately monitored to ensure their efficacy and safety.

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INTRODUCTION

With the advent of technology started by the Industrial Revolution, people in the United States have had fewer reasons to be physically active. In the last 50 years there has been a rise in the use of television (with 10% of households having a television set in 1950 compared to 98% of households in 2001), a rise in the number of desk jobs (with 26.8% of Americans today having an occupation that has little to no activity compared to 12.6% in 1950), and decreases in the levels of daily exercise (with only 26.2% of Americans meeting the recommended 30 minutes of moderate physical activity at least 5 times per week or vigorous activity for 20 minutes at a time at least 3 times per week; Brownson et al. 2004). As a result, obesity and the health problems associated with it have risen at a dramatic rate in the USA over the past few decades, with obesity rising from 13.3% of the population in 1960 to 30.4% in 2002 (Want et al. 2007). With this increase in problems related to physical health, people are starting to modify their lifestyles to include more exercise, or to change their diet in an effort to fight against this growing epidemic.

One area of dietary change has been the rise in the use of herbal supplements and natural products. Today, these natural products, specifically herbal supplements, have become increasingly popular with the general public as they look to help improve their health. The total sales of herbal and botanical dietary supplements in the United States increased 5.5% from 2011 to 2012, the ninth year in a row that sales have increased (Lindstrom et al. 2013). Natural products from plants, and indeed plants themselves, have been used to treat illnesses for thousands of years. Many people believe in the effectiveness of herbal supplements and use them regularly for such things as memory enhancement, physical well-being, or to promote a more

positive mood. With the growth of the industry, herbal supplement companies have reaped the rewards. It has become a multi-billion dollar industry and is predicted to grow to an almost $60 billion dollar industry by 2015 (DaVanzo et al. 2009). With this much popularity, one might expect that there would be ample scientific research to back up the effectiveness of herbal supplements. However, such research has proven to be inconclusive. Most herbal supplements are not fully regulated by the Food and Drug Association (FDA). Herbal supplements are placed in a separate group from food or medical products. Unlike food or medicine, the manufacturers themselves, not the FDA, is responsible for determining whether a supplement is effective, what is included in the supplement, and whether it is safe. Only after the herbal supplement is on the market does the FDA get involved, and they are only responsible for taking supplements off the market that are reported to have adverse side effects through reports by consumers or researchers (Newmaster et al. 2013). So what is contained within the supplement or how herbals are processed may not necessarily be what is presented by the manufacture. Because the industry is sparsely regulated, supplements may contain materials other than plant matter, such as bacterial cell components. The purpose of this study was to examine a selection of common herbal supplements for the presence of bacterial cells. Because supplements may be dried or even irradiated, rather than look for living cells, I chose to test for the presence (and type) of bacterial DNA, as an indication of past bacterial contamination. Herbal supplements were chosen from those that were easily available to the public in local grocery stores in Oxford, MS, and included Echinacea, Golden Seal Extract, Korean Panax Ginseng, Ginkgo Biloba, St. John's Wort, and Ginger Root. These supplements cover a range of potential benefits.

Echinacea (*Echinacea purpurea)* is a purple flowering plant native to the U.S. Midwest and has been used in the United States since the early 1800s. Its therapeutic uses have changed

over its long history from external application on wounds such as burns, snake bites, and cuts to treatment for internal ailments such as pain, stomach cramps, and coughs (Hostettmann 2003). Today the plant is used as a preventative measure for colds, the flu, and other illnesses as it is thought to boost the immune system. However, the effectiveness of its consumption has been found to be varied (see studies by Shah et al. 2007 and Barrett et al. 2010).

Golden Seal (*Hydrastis canadensis)* is a small green plant with a white flower that is native to southeastern Canada and the northeastern United States. It is thought to have been used prior to the 1700s by Native Americans, but was first recorded by Hugh Martin in 1793 when it was used as a yellow dye. Its first medicinal use was registered by Benjamin Smith Barton in 1798, who described its use in treating native Cherokee Indians who had cancer (Koffler et al. 1957). Today, Golden Seal is used to treat a myriad of ailments such as the common cold, influenza, menstrual disorders, and traveler's diarrhea. While there are some studies that speak to the effectiveness of a compound, berberine, found in it, (Kong et al. 2012), there have been few studies to prove the effectiveness of the plant itself. However, there are well-documented negative side effects when taken with other medications and in pregnant women and children (Mahandy et al. 2001)

Korean Panax Ginseng (*Panax ginseng)* is a small green plant with red berries and is found both in North America and in eastern Asia. It has likely been used by people since before recorded history. It is first reported in the book “Shennong Bencao Jing” (Shennong’s Herbal) by Tao Hongjing in 502-557 A.D. Its roots were seen as a cure-all in ancient times, even with legends being told of its power (Yun 2001). Today ginseng is used much the same as it was thousands of years ago: as a cure-all for general illnesses such as depression, chronic fatigue,

cancer, diabetes, fever, asthma, and even hangovers. As with many of the other herbal supplements, the results for its effectiveness are widely varied (Kiefer et al. 2003).

Ginkgo Biloba (*Ginkgo biloba)* is a large tree native to China that is labeled as a "living fossil." It was thought to be extinct before it was discovered again by the German scientist Englebert Kaempfer in 1691. It was brought to the Americas in 1778 by William Hamilton and has grown to be one of the most popular herbal supplements used today (Beek 2001). Ginkgo biloba has been used to improve memory and treat diseases associated with it. The effectiveness of the herbal supplement has been evaluated and some positive effects have been recorded for certain groups of people, specifically the elderly or people ages 50-59 who suffer from dementia. However, it seems to show no positive effect against other memory related diseases (Oken et al. 1998, Rigney et al. 1999).

St. John's Wort (*Hypericum perforatum*) is a yellow, flowering plant found in many subtropical climates throughout the world. The plant was first documented by the General Proscurides in the 1st century AD. In ancient times it was used in "magic potions" and for general illness (Pöldinger 2000). Today, St. John's Wort is used to enhance a positive mood to fight against depression. As with the other supplements examined, this herb's efficacy is also inconclusive (Hypericum Depression Trial Study Group, Gaster et al. 2000).

Ginger Root (*Zingiber officinale*) has more of a culinary history than a medicinal one. It was first cultivated in South East Asia and was first recorded in the 4th century BC in the writings of the Hindu epic, Mahabharata. It was used in soups, meats, and other dishes as a spice and was one of the most commonly traded spices during the middle ages (Benzie 2011). In terms of its usefulness in medicine, it is used today to help with nausea, inflammatory diseases, and

motion sickness, with there being variable data in terms of its efficacy (Visalyaputra et al. 2002, Chaiyakunapruk et al. 2006).

As stated above, most of the herbal supplements examined in this study have been found to be highly variable when it comes to their efficacy. One potential explanation for this variability is that bacteria or components of bacteria could be contributing to the health benefits, and that bacterial populations vary from one batch of supplement to another. This has recently been shown for Echinacea (Pugh et al. 2013), one of the supplements examined here. In this study, as well as testing for the presence of bacterial DNA, the type of bacterial DNA present was identified through partial 16S rRNA gene sequencing in order to identify possible species that could be contributing to the efficacy of the supplement. Such sequencing could also reveal the past presence of potential pathogens, suggesting health risks when consuming these materials.

METHODS

The samples of commercial herbal supplements were bought from the Oxford, MS, Walmart store on February 14, 2013. Six types of herbal supplements were obtained, all from the Spring Valley brand. Samples are described in Table-1 and were Echinacea (labeled "whole herb"), Golden Seal Extract (labeled "natural"), Korean Panax Ginseng (labeled "standardized extract"), Ginkgo Biloba (labeled "standardized extract"), St. John's Wort (labeled "standardized extract"), and Ginger Root (labeled "whole herb").

For each herb type, one capsule was opened and 0.05g of dried material was weighed and transferred to a sterile 2 mL DNA extraction tube. The Echinacea and Ginger Root samples were rehydrated with 150µL of sterile H_2O. The Golden Seal Extract, Korean Panax Ginseng, Ginkgo Biloba, and St. John's Wort samples were rehydrated with 50 µL of sterile H_2O (less water was needed to hydrate these samples). DNA was extracted using a PowerPlant Pro DNA Isolation Kit (MO BIO Laboratories, INC., Carlsbad, CA) following the manufacturer's instructions. After extraction, the extracted DNA was cleaned using the Power Clean DNA Clean-Up Kit (MO BIO Laboratories, INC, Carlsbad, CA), again in accordance with the manufacturer's instructions. Cleaned extractions were electrophoresed through agarose gels (210 V, 25 minutes) to determine whether DNA was present. Gels were stained with ethidium bromide for 10 minutes, rinsed, and visualized on a Kodak Gel Logic 200 Imaging System. DNA samples were stored in a freezer (-20ºC) until subsequent amplification. Because samples did not consistently yield DNA (see Results), this extraction procedure was repeated up to five times until satisfactory quantities of DNA were obtained.

Table-1: Herbal supplements analyzed for the presence of bacterial DNA during the study. All information was obtained from the supplement bottle (Spring Valley)

Supplement Name	Main Ingredient(s)	Amount (mg)	Other Ingredient(s)	Benefit as labeled
Echinacea	*Echinacea purpurea* (aerial part)	760	Gelatin, Medium Chain Triglycerides	Support healthy immune function.
Golden Seal Extract	*Hydrastis canadensis*	400	Rice flour, Gelatin	Support healthy immune function.
Korean Panax Ginseng	*Panax ginseng* (root) standarized to contain 7% ginsenosides	100 7	Maltodextrin, Gelatin, Cellulose (plant origin), Silica, Vegetable Magnesium Stearate	Supports physical performance
Ginkgo Biloba	*Ginkgo biloba* (leaf) Standardized to contain 24% Ginkgo Flavone Glyscosides	120 28	Rice Powder, Gelatin, Vegetable Magnesium Stearate, Silica	Memory support
St. John's Wort	*Hypericum perforatum* (aerial) Standardized to contain 0.3% Hypericin	300 0.9	Maltodextrin, Gelatin, Magnesium Silicate, Vegetable Magnesium Stearate, Silica	Promotes positive mood
Ginger Root	*Zingiber officinale* (root)	550	Gelatin, Vegetable Magnesium Stearate, Silica	For Occasional Motion Sickness

Amplification of 16S ribosomal RNA genes

Samples were amplified with primers for bacterial 16S ribosomal RNA (16S rRNA) genes to determine if bacterial DNA was present. Because of very low yields of bacterial DNA from each sample, amplification consisted of a two-step procedure in which all potential DNA was initially amplified, followed by selective amplification of non-chloroplast, bacterial specific DNA. Amplifications were generally carried out following the procedures of Jackson et al. (2001). A 48 µL amplification mixture was prepared containing 2 mM MgCl2 buffer solution, 0.2 mM deoxynucleotide triphosphates (dNTPs), 0.4 µM Bac 8F Primer, 0.4 µM Univ 1492 Primer, and 1.0 U Taq DNA polymerase. The 48 µL of PCR mixture was mixed with 2 µL of sample DNA. A positive control, containing previously confirmed E. coli bacterial DNA and a negative control, containing no template DNA, were routinely run to verify experimental procedures. Amplification reactions were carried out on a MyCyler thermal cycler (Bio-Rad Industries, Hercules, CA) using a program cycle of 95ºC for 2 min, 26 cycles of 95ºC for 1 min; 45ºC for 1 min; and 72ºC for 7 min; followed by an elongation step of 72ºC for 7 minutes. Products of the amplification reaction were run on agarose gels, as previously described, in order to confirm presence of DNA.

To specifically target the bacterial community, these amplification products underwent a secondary amplification using primers that do not amplify chloroplast 16S rRNA genes and differentially amplify bacterial and mitochondrial rRNA (Chelius and Triplett 2001). Successful PCR products from the initial amplification were amplified using the same mixture as above except instead of the Bac 8F primer, a Bac 799F primer was used. Reactions were run in the thermal cycler using the following cycling program: 95ºC for 3 min, 30 cycles of 94ºC for 20 sec; 50ºC for 40 sec; and 72ºC for 40 sec; followed by an elongation step of 72ºC for 7 min.

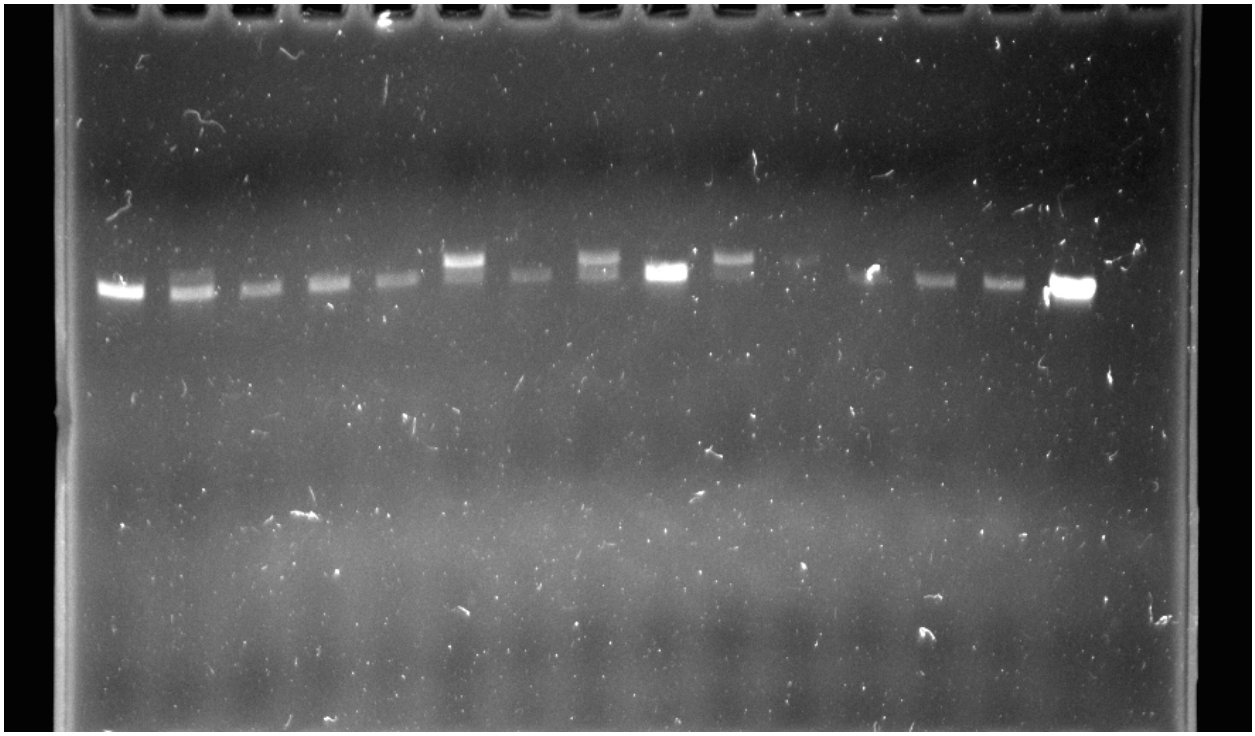
amplified bacterial DNA and a larger fragment of the amplified plant mitochondrial 16S rRNA gene (Chelius and Triplett 2001). The bacterial band from each sample was extracted from the gel matrix by cutting out the lower bad (see Figure-1) for each electrophoresed product. These extracted amplification products from these reactions were sequenced using high throughput 454 next generation sequencing.

Sequencing and data analysis

PCR samples were sent for high throughput next generation (454) sequencing to Molecular Research DNA, Shallowater, TX, for analysis. Amplicon pyrosequencing (bTEFAP) was performed using 16S universal Eubacterial primers. A single-step 30 cycle PCR using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) were used under the following conditions: 94ºC for 3 minutes, followed by 28 cycles of 94ºC for 30 seconds; 53ºC for 40 seconds and 72ºC for 1 minute; after which a final elongation step at 72ºC for 5 minutes was performed. Following PCR, product samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Samples were sequenced utilizing Roche 454 FLX titanium instruments and reagents and following manufacturer's guidelines.

The Q25 sequence data derived from the sequencing process was processed using a proprietary analysis pipeline (www.mrdnalab.com , MR DNA, Shallowater, TX). Sequences were depleted of barcodes and primers then short sequences < 200bp were removed, sequences with ambiguous base calls were removed, and sequences with homopolymer runs exceeding 6bp were removed. Sequences were then denoised and chimeras removed. Operational taxonomic units were defined after removal of singleton sequences, clustering at 3% divergence (97%

Figure-1: Positive samples after amplifying using 8-1492 primers followed by 799-1492 primers. The presence of an upper band in each sample indicates mitochondrial DNA, while the lower band is bacterial. This was later excised to yield only bacterial 16S DNA. The final two wells show positive and negative controls, respectively.



similarity). (Dowd et al. 2008; Swanson et al. 2011). OTUs were then taxonomically classified using BLASTn against a curated GreenGenes database (DeSantis et al. 2006).

RESULTS

DNA Extraction and Amplification

The first attempt at DNA extraction gave just one positive result (Golden Seal Extract) according to the visible presence of DNA on agarose gels, and subsequent repeated extractions showed no visible DNA extracted from most samples. However, amplification using the 8-1492 primer set was still done on all samples to check for presence of amplifiable bacterial DNA not detected by initial electrophoresis analysis. PCR gave much more consistent positive results suggesting that bacterial DNA was present but not in high concentrations inside many of the supplement samples. Thus, amplifiable bacterial DNA was eventually obtained from Echinacea, Golden Seal Extract, Korean Panax Ginseng, St. John's Wort and Ginger Root. A total of 14 positives were obtained through five extractions: one from Echinacea, three from Golden Seal, four from Korean Panax Ginseng, four from St. John's Wort, and two from Ginger Root (Figure-1). The amplicons from the 8-1492 reactions were used as the template for a secondary 799-1492 amplification to exude chloroplast DNA, all of which yielded positive results. Thus, after five extractions with subsequent PCRs, a total of 14 out of 42 samples tested positive for bacterial DNA, or 33%. There were no positive results found for Ginkgo Biloba and therefore no Ginkgo Biloba samples were sent for sequencing analysis.

Taxonomical Analysis

Upon receiving the sequencing results from Molecular Research DNA, Shallowater, TX, data were sorted in order to exclude any bacterial DNA making up less than 1% of the sample so that the focus of analysis could be on dominant bacterial populations. The majority of the sequences identified in Echinacea indicated potentially pathogenic bacteria (Table-2). Echinacea

Table-2: Dominant bacterial species identified in samples of the herbal supplement Echinacea as revealed by next generation 16S rRNA gene sequencing.

Echinacea	**% of total bacterial DNA sequences obtained from sample**
Salmonella enterica	40.21
Lactobacillus spp.	18.17
Salmonella paratyphi a	9.70
Shingella sonnei	7.70
Echerichia albertii	6.07
Escherichia_shigella salmonella enterica_subsp._enterica_serovar_typhi	4.10
Echerichia_Shigella spp.	3.23
Cronobacter spp.	2.75
Clostridium spp.	1.09

contained a high proportion of DNA sequences from bacteria *Salmonella enterica, Salmonella paratyphi a, Shingella sonnei,* and *Echerichia albertii*, as well as other potentially pathogenic species that could not be determined specifically such as *Echerichia_Shigella spp., Cronobacter spp.,*and *Clostridium spp.* Nonpathogenic bacteria detected in the Echinacea sample included *Lactobacillus spp.*

Salmonella enterica was also the proportionally most abundant species detected in both of the Korean Panax Ginseng samples (Table-3). As with Echinacea, *Lactobacillus spp.* was the second most common sequence type found in these samples. Korean Panax Ginseng also contained DNA from other potential pathogens that were found in the Echinacea sample, with the exception of *Cronobacter spp.* which was not detected in one of the Korean Panax Ginseng samples (Table-3). This sample did contain sequences related to other potential pathogens such as *Acinetobacter johnsonii* and *Salmonella serovar heldelberg enterica*.

The Ginger Root samples were more variable (Table-4). The first sample was dominated, as was Echinacea and Korean Panax Ginseng, by *Salmonella enterica* and *Lactobacillus spp.* However, for the second sample *Enterobacter asburiae* and *Leclercia adecarboxylata* were the most prevalent sequences identified (Table-4). Other potential pathogens found in both Ginger Root samples that were not found in either Echinacea or Korean Panax Ginseng were *Enterobacter asburiae, Leclercia adecarboxylata, Enterobacter hormaechei, Klebsiella variicola, Erwinia Escherichia hermannii, and Klebsiella pneumonia* (Table-4).

Even greater variability between samples was apparent for Golden Seal, and these samples were the most variable in the bacterial DNA present of all of the samples tested. The first Golden Seal sample followed the same pattern as many of the other supplements, with *Salmonella enterica* being the most abundant sequence detected, followed by *Lactobacillus spp.*

Table-3: Dominant bacterial species identified in samples of the herbal supplement Korean Panax Ginseng as revealed by next generation 16S rRNA gene sequencing.

Korean Panax Ginseng 1	**% of total bacterial DNA sequences obtained from sample**	**Korean Panax Ginseng 2**	**% of total bacterial DNA sequences obtained from sample**
Salmonella enterica	34.46	*Salmonella enterica*	41.58
Lactobacillus spp.	15.45	*Lactobacillus spp.*	17.19
Acinetobacter johnsonii	9.11	*Salmonella paratyphi a*	8.92
Salmonella paratyphi a	8.77	*Shigella sonnei*	8.00
Shigella sonnei	8.20	*Escherichia albertii*	4.95
Escherichia albertii	4.53	*Escherichia_shigella spp.*	4.11
Escherichia_shigella salmonella enterica_subsp._enterica_serovar_ typhi	3.48	*Enterobacter cowanii*	3.58
Enterobacter cowanii	2.90	*Escherichia_shigella salmonella enterica_subsp._enterica_serovar_ typhi*	3.56
Escherichia_shigella spp.	2.57	*Clostridium spp.*	1.20
Salmonella serovar heidelberg enterica	1.68	*Cronobacter spp.*	1.09
Clostridium spp.	1.46		

Table-4: Dominant bacterial species identified in samples of the herbal supplement Ginger Root as revealed by next generation 16S rRNA gene sequencing

Ginger Root 1	**% of total bacterial DNA sequences obtained from sample**	**Ginger Root 2**	**% of total bacterial DNA sequences obtained from sample**
Salmonella enterica	20.32	*Enterobacter asburiae*	26.17
Lactobacillus spp.	13.30	*Leclercia adecarboxylata*	19.51
Enterobacter cowanii	9.26	*Clostridium spp.*	10.09
Salmonella paratyphi a	8.78	*Enterobacter hormaechei*	8.95
Shigella sonnei	6.38	*Enterobacter cowanii*	5.72
Enterobacter hormaechei	5.81	*Klebsiella pneumoniae ynucc0237*	4.37
Escherichia_shigella salmonella enterica_subsp._enterica_serovar_typhi	4.25	*Klebsiella variicola*	2.55
Clostridium spp.	3.85	*Erwinia escherichia hermannii*	2.19
Leclercia adecarboxylata	2.93	*Clostridium algidixylanolyticum*	2.13
Enterobacter asburiae	2.80	*Salmonella enterica*	1.61
Escherichia albertii	2.69	*Lactobacillus spp.*	1.51
Escherichia citrobacter koseri	2.61	*Salmonella paratyphi a*	1.35
Escherichia_shigella spp.	2.50	*Escherichia_shigella salmonella enterica _subsp._enterica_serovar_typhi*	1.20
Clostridium algidixylanolyticum	1.43		
Klebsiella variicola	1.32		
Erwinia escherichia hermannii	1.02		
Klebsiella pneumoniae ynucc0237	1.02		

(Table-5). However, the bacterial community in the second sample of Golden Seal was almost entirely *Trabulsiella citrobacter rodentium* (79% of sequences recovered) with much lower proportions from its next most abundant species (*Shigella sonnei* at 3.80%) and *Lactobacillus spp.* at 3.19%). Bacteria that appeared to be unique to these samples included *Pantoea ananatis* and *Pantoea agglomerans* in the first Golden Seal sample tested, as well as the previously mentioned *Trabulsiella citrobacter rodentium* and *Trabulsiella citrobacter farmeri* in the second (Table-5).

St. John's Wort also showed a high level of variation between samples. As with one of the Golden Seal samples, one sample of St. John's Wort was dominated by sequences identified as *Trabuisiella citrobacter rodentium* (67% of all sequences recovered; Table-6). The second St. John's Wort sample was more typical of the supplements in general, with *Salmonella enterica* and *Lactobacillus spp.* being the most prevalent bacterial sequences present. While there were no unique bacteria in the first St. John's Wort sample, the second contained *Acinetobacter johnsonii, Salmonella serovar Virchow enterica, and Ervina cyrpripedii*, which were not detected in other samples, as well as sequences identified as mitochondrial sequences from O*ryza glaberrima* (African rice; Table-6).

In summary, all of the samples sequenced contained sequences related to *Salmonella enterica* and *Lactobacillus spp.* (Table-7). Six out of the nine samples examined had *Salmonella enterica* as the most abundant species, the exceptions being the second Ginger Root sample, the second Golden Seal sample and the first St. John's Wort sample, with the Golden Seal and St. John's Wort samples both having *Trabulsiella citrobacter rodentium* as their most abundant

Table-5: Dominant bacterial species identified in samples of the herbal supplement Golden Seal as revealed by next generation 16S rRNA gene sequencing

Golden Seal 1	**% of total bacterial DNA sequences obtained from sample**	**Golden Seal 2**	**% of total bacterial DNA sequences obtained from sample**
Salmonella enterica	46.32	*Trabulsiella citrobacter rodentium*	78.73
Lactobacillus spp.	11.11	*Shigella sonnei*	3.80
Shigella sonnei	10.55	*Lactobacillus spp.*	3.19
Salmonella paratyphi a	8.22	*Enterobacter hormaechei*	1.87
Escherichia_shigella salmonella enterica_subsp._enterica_serovar _typhi	3.80	*Salmonella enterica*	1.32
Enterobacter cowanii	3.04	*Klebsiella variicola*	1.32
Escherichia_shigella spp.	2.21	*Escherichia_shigella spp.*	1.26
Escherichia albertii	2.13	*Trabulsiella citrobacter farmeri*	1.09
Pantoea ananatis	1.61		
Pantoea agglomerans	1.38		
Cronobacter spp.	1.21		
Clostridium spp.	1.19		

Table-6: Dominant bacterial species identified in samples of the herbal supplement St. John's Wort as revealed by next generation 16S rRNA gene sequencing

St. John's Wort 1	**% of total bacterial DNA sequences obtained from sample**	**St. John's Wort 2**	**% of total bacterial DNA sequences obtained from sample**
trabulsiella citrobacter rodentium	66.33	*Salmonella enterica*	24.06
salmonella enterica	4.68	*Lactobacillus spp.*	12.61
lactobacillus spp.	4.07	*Mitochondria oryza glaberrima_(african_rice)*	12.47
shigella sonnei	3.67	*Pantoea agglomerans*	6.97
enterobacter hormaechei	2.16	*Pantoea ananatis*	6.08
trabulsiella citrobacter farmeri	2.09	*Salmonella paratyphi a*	5.57
escherichia_shigella spp.	1.60	*Escherichia albertii*	4.00
klebsiella variicola	1.55	*Shigella sonnei*	3.93
acinetobacter johnsonii	1.41	*Clostridium spp.*	2.56
escherichia citrobacter koseri	1.13	*Escherichia_shigella salmonella enterica_subsp._enterica_serovar_typhi*	2.46
		Enterobacter hormaechei	1.81
		Acinetobacter johnsonii	1.61
		Enterobacter cowanii	1.57
		Salmonella serovar virchow enterica	1.30
		Erwinia cypripedii	1.03

Table-7: Summary of the bacterial component present in herbal supplements. A shaded square indicates that the specific bacterium was identified in that sample using next generation 16S rRNA gene sequencing. The numbers inside the shaded boxes represent the percentage of the sample that contained that particular species of bacteria. **E** = Echinacea; **KPG** = Korean Panax Ginseng; **GR** = Ginger Root; **GS** = Golden Seal; **SJW** = Saint John's Wort.

Bacteria (# of samples containing it)	**% present to at least 1% of Sample**								
	E	**KPG 1**	**KPG 2**	**GR 1**	**GR 2**	**GS 1**	**GS 2**	**SJW 1**	**SJW 2**
Salmonella enterica (9)	40.2	34.5	41.6	20.3	1.6	46.3	1.3	4.7	24.1
Lactobacillus spp. (9)	18.2	15.5	17.2	13.2	1.5	11.1	3.2	4.1	12.6
Shigella sonnei (8)	7.7	8.2	8.0	6.4		10.6	3.8	3.7	3.9
Salmonella paratyphi a (7)	9.7	8.8	8.9	8.8	1.4	8.2			5.6
Escherichia_shigella spp. (7)	3.2	2.6	4.1	2.5		2.2	1.3	1.6	
Clostridium spp. (7)	1.1	1.5	1.2	3.9	10.1	1.2			2.6
Escherichia_shigella salmonella enterica_subsp._enterica_serovar_typhi (6)	4.1	3.5	3.6	4.3	1.2	3.8			2.5
Escherichia albertii (6)	6.1	4.5	5.0	2.7		2.1			4.0
Enterobacter cowanii (6)		2.9	3.6	9.3	5.7	3.0			1.6
Enterobacter hormaechei (5)				5.8	9.0		1.9	2.2	1.8
Klebsiella variicola (4)				1.3	2.6		1.3	1.6	
Cronobacter spp. (3)	2.8		1.1			1.2			
Acinetobacter johnsonii (3)		9.1						1.4	1.6
Clostridium algidixylanolyticum (2)				1.4	2.1				
Leclercia adecarboxylata (2)				3.0	19.5				
Erwinia escherichia hermannii (2)				1.0	2.2				
Klebsiella pneumoniae ynucc0237 (2)				1.0	4.4				
Escherichia citrobacter koseri (2)				2.6				1.1	

Bacteria (# of samples containing it)	**% present to at least 1% of Sample**								
	E	**KPG 1**	**KPG 2**	**GR 1**	**GR 2**	**GS 1**	**GS 2**	**SJW 1**	**SJW 2**
Enterobacter asburiae (2)				2.8	26.2				
Pantoea agglomerans (2)						1.4			6.7
Pantoes anatis (2)						1.6			6.1
Trabulsiella cirtrobacter farmeri (2)							1.1	2.1	
Trabulsiella citrobacter rodentium (2)							78.7	66.3	
Salmonella servar Heidelberg enterica (1)		1.7							
Mitochondria oryza glaberrima_(african_rice) (1)									12.50
Salmonella serovar virchow enterica (1)									1.3
Ervinia cyrpripedii (1)									1.0
Total number of species attributing to at least 1% of the sample	9	11	10	17	13	12	8	10	15

species. The most diverse sample tested was the first Ginger Root sample (17 different species that were present at proportions of >1% of the sequences obtained) and the least diverse sample was the second Golden Seal sample (eight different species present at proportions of >1% of the total sequences obtained).

DISCUSSION

With the rise in the number of sedentary occupations, the prevalence and popularity of unhealthy foods, and the rise in the number of preventable exercise/diet related medical conditions, professionals and ordinary citizens alike have begun to voice their concerns to the general public. Here in Mississippi programs like “Let’s Go Walkin’ Mississippi” and organizations like “Healthworks” have been established to both raise awareness and encourage activity in an effort to reduce the number of very preventable diseases. But this is not restricted to Mississippi alone. According to a Gym, Health & Fitness Clubs market research report done by the IBISWorld, nationally, people are increasingly turning to weight training and fitness programs to alleviate these growing problems (with an increase of annual memberships from 46.4 million memberships in 2002 to more than 52.6 million by 2013; IBISWorld, 2014). Others are looking to their diet for answers, and this is one of the major reasons herbal supplements have become so popular in the United States.

Companies have taken advantage of the meteoric rise of the health awareness trend for their own substantial gain. The herbal supplement industry is now a multi-billion dollar industry with almost 20% of people in the United States consuming them every year (Gahche et al., 2011). It is unsettling that a product that is so profitable and so widely used is not fully regulated by the FDA. While there are measures in place to take a harmful product off the market, there is no requirement for herbal supplement companies to tell their customers what the product actually contains, or if what they say its effects will be are completely accurate. Because of this the general public really has no idea of the process of getting an herbal supplement from the ground to the bottle, and what it is they are actually consuming. In this study I examined the potential

bacterial composition of herbal supplement pills and hoped to get an idea of possible bacteria that contribute to its effectiveness. I also wanted to determine if this product needs more oversight in its harvesting, processing, or distribution. By examining these products bacterial composition for both beneficial and potentially harmful bacteria one can better weigh the risk/reward of consuming each product.

There are numerous ways to approach the data obtained. At one level, one could use this study as a taxonomical survey of all of the bacterial species found in each supplement. However, focusing on the most proportionally abundant bacteria rather than all of the species detected is more useful, since these would be the bacteria that would most affect the consumer. These bacteria could potentially be beneficial or harmful to an individual. From these results, there do not seem to be any bacteria present in significant proportions that are recognized as contributing to the supposed effects of the supplements; at least not in the way we currently understand and classify bacteria. Rather, most of the common bacteria that were present are actually pathogenic. The most abundant five species found were *Salmonella enterica, Salmonella paratypi a, Shigella sonnei, Escherichia_Shigella spp, Clostridium spp.,* and *Lactobacillus spp.* Most of these species are regarded as at least opportunistic pathogens; however, *Lactobacillus* has been found to be beneficial in some circumstances.

Salmonella enterica is regarded as one of the most common bacterial causes of food poisoning, causing mild to severe diarrhea, fever, and cramps. Although most cases last between 4 to 7 days, there are instances where the bacteria can spread throughout the body through the blood stream and cause more severe symptoms. According to the Centers for Disease Control and Prevention (CDC), almost 400,000 Americans get salmonellosis each year, with an estimated number of actual cases being thirty times greater or more because of misdiagnosis or it

not being reported (CDC, 2014). From these cases more than 400 people die annually. The most common way for *Salmonella* to contaminate its sources is via animal (or human) feces. How *S. enterica* was introduced into the herbal sample could potentially be explained through the use of animal waste fertilizers during the growing process. The bacteria would remain on the plant material if not thoroughly washed during later processing. Salmonella could also be introduced as a contaminant from workers or the environment during the handling or sorting process. Because herbal supplements are not regulated by the FDA, we really do not know how the plant material is processed. It is entirely possible that workers handle the products without the use of gloves or other protective measures. Either way, every sample contained *S. enterica,* which raises some concerns regarding the widespread prevalence of this bacterium in herbal supplements.

Salmonella paratyphi a is a bacterium endemic to the human gastrointestinal tract and is associated with the disease parathyroid fever. This disease, although more common in other parts of the world (6 million global cases a year), still has an estimated 100 cases per year in the United States. The symptoms include sustained fever, headache, and abdominal pain. The bacteria are usually transferred through people who are already infected or have poor hygiene and transfer it to another host or to food that is ingested by a potential host (Brunette et al. 2014). Just as with *S. enterica*, this bacterium is most commonly associated with the human digestive tract. Therefore, it likely entered the herbal supplements during worker handling and processing. *S. paratyphi a* was found in seven of the samples examined.

Shigella sonnei is a species of bacteria that is the leading cause of shigellosis (70% of cases) in the United States. The only known natural host for this bacterium is in the human gastrointestinal tract. There are more than 14,000 cases of shigellosis a year. *Shigella sonnei* is

usually transferred through individuals who do not practice basic hygiene and/or hand washing techniques (CDC, 2014). The fact that this bacterium only comes from the human gastrointestinal tract is unsettling. This tells us that contamination had to have come from human contact whether by directly handling of the product or indirectly by the bacteria coming from an environment that was already contaminated, such as a drying rack or conveyer belt. *S. sonnei* was found in all but one of the samples.

According to the CDC, the genera *Escherichia* and *Shigella* are found predominately in the human gastrointestinal tracts (with *Escherichia* also being present in other homeothermic animals). Although many species in *Escherichia* and *Shigella* are harmless, both groups of bacteria can be associated with bacterial pathogens such as one of the most recognizable and most common source of urinary tract infections and food poisoning *E. coli*. Because both genera are present in the gastrointestinal tract and are transferred through contamination via fecal matter directly to the plant or secondarily because of poor hygiene, there seem to be preventable ways to keep these high abundances of bacteria away from the product. As with most of the other bacteria, sources of contamination could have come during the growing or processing phases of production. *Escherichia_Shigella spp.* was found in seven of the samples.

The genus *Clostridium* contains many different species, including those that are neutral and pathogenic to humans. Two pathogens of interest present in the samples were *C. difficile* (which can cause diarrhea and infections of the large intestine) and *C. perfringens* (a potential cause of food poisoning). *C. difficile* is found in many different environments, including animal intestines and soil, and virulent strains can be produced following extensive use of antibiotics. Once it becomes virulent, this bacteria can cause antibiotic-associated diarrhea, which can range from being mild to severe. *C. perfringens* is also found in many environments and is another one

of the main causes of food poisoning in the United States, causing an estimated million cases a year. It typically causes diarrhea within 24 hours of contraction and usually lasts no more than 24 hours.

One important unifying characteristic of *Clostridium* is that bacteria in this group emit a neurotoxin that is most often associated with its harmful effects. Although rarely lasting long enough to cause disease and not normally associated with plants, this group has been found to release the neurotoxin in stressed environments, which could persist and cause disease after the bacteria is gone (Yule et al. 2006). Spores produced by *Clostridium* have also been found to last months and even years after formation in unfavorable environments (Setlow et al. 2007). These persistent spores can reanimate in a suitable environment and begin producing neurotoxins if stressed. With *Clostridium* potentially coming from animal intestines or from the surrounding soil environment there is no way of knowing where it was introduced during the production of the herbal supplement, but it was found in seven of the samples examined.

Although all of the previously discussed bacteria were at least opportunistic pathogens, *Lactobacillus* has been found to be beneficial to its host in some species and in some circumstances. *Lactobacillus* is a group of bacteria widely found in animals' gastrointestinal tracts. While *Lactobacillus* can be found on plants, they appear to be present only as transients as part of a cycle which begins in the intestine and moves out via defecation and enters the host again via ingestion. Most species of *Lactobacillus* are associated with beneficial or neutrally affecting bacteria (Klaenhammer, 2006). Consumption of particular species of *Lactobacillus* as a probiotic has been found to treat many different ailments. For example some trials have found a *Lactobacillus* probiotic may decrease adverse effects associated with lactose-intolerant patients (Almeida et al. 2012), resolve pains and promote normalization of stool for people with irritable

bowel syndrome (Neidzielin et al. 2001), and if taken before infection may help prevent enteropathic *Escherichia coli* infections (Michail et al. 2002). Because this bacteria is common in many animal (including human) gastrointestinal tracts, both of the explanations of its origin stated above are valid: the use of organic fertilizers, or through contamination during processing. However, for *Lactobacillus*, there is less of a concern in terms of being pathogenic because most of the group's species are harmless, if not beneficial to its hosts.

Other bacterial species of note include *Trabulsiella Citrobacter rodentium* and *Oryza glaberrima*. *Trabulsiella Citrobacter rodentium* was found in extremely high proportions in Golden Seal 2 and St. John's Wort 1 and is associated with the distal colon of mice (Luperchio et al. 2001), which is interesting because the only source of this bacterium would be from mice feces. The presence of this particular bacterium means that somewhere in the process mice were present. This could have been just from the fields where the herbs are collected or, more interestingly, there could have been mice present in the factory where they were processed or warehouse where they were stored. This raises even more questions about the how possible contaminants could get into herbal supplements, such as during the drying process or during storage.

O*ryza glaberrima* sequences were found in relatively high proportions in St. John's Wort 2 (making up 12.47% of the sample), but is actually a sequence from the mitochondrion of rice (Linares 2002). It is interesting that this would be found in significant proportions because the methodology of the study specifically selected for bacterial DNA. The presence of the mitochondrial DNA suggests that the either/both the sorting and/or the sequencing process was not 100% selective for just bacteria. However, the fact that it was present in only one sample is encouraging as it relates to accuracy of the study.

While the results of this study quite clearly demonstrate potential bacterial contamination of many herbal supplements, it may be impossible for much of this contamination to be avoided. Many of the bacterial groups and species detected are very common and bacteria are the most abundant organisms found on the planet and are present on almost every surface. As such, contamination may be unavoidable in some circumstances. One thing to consider is that for this study positive identification of a bacterium only indicates the presence of its DNA, not actual living and viable bacteria. Because of this, one cannot assume that herbal supplements on grocery store shelves contain living *S. enterica* or *Shigella sonnei* bacteria in them, but at some point in time they did. The bacteria may have been previously killed either through sterilization efforts or in some other phase of processing the supplements. Knowing the prevalence of bacteria, it is interesting that no positive results were collected with Ginkgo Biloba. It is difficult to speculate why no bacteria was detected in the Ginkgo Biloba samples as they were tested eight times (two samples through four extractions) for the presence of DNA without success. However, this may suggest that it is indeed possible to eliminate the presence of bacteria in the samples in significant, detectable portions.

Another point to keep in mind about the detected bacteria is that while there were some supplements whose replicate samples were relatively similar, in general there was variability in the proportions of bacterial species within the same types of samples. Some of this variation is skewed by the samples containing *Trabulsiella Citrobacter rodentium*, but even without this sequence there is significant variability in some of the samples. For example, the three most abundant bacterial sequence types for the two Golden Rod samples were completely different. On the other end of the spectrum the Korean Panax Ginseng samples were very similar in their composition, with the exception of just a few species of bacteria. This does mean that even if

there were pathogenic bacteria present in a sample that had somehow survived to the supplement bottle, they may not be present in all of the capsules within that bottle. It would be interesting to compare the bacterial compositions of all of the capsules in a bottle of the same herbal supplement in order to understand the intra-bottle variability. This may help to identify more bacterial species that could possibly be contributing to the effects of the herbal supplements. Similarly, one could examine the same herbal supplement from different manufacturers (as all of the samples observed in this study were from the same brand) in order to determine whether different companies are more prone to the presence of certain bacteria. Testing for the presence of bacteria at different stages of processing would also be interesting, and might suggest when and where possible bacterial contaminants entered the chain.

Given the findings of this study, it is unsettling that there is little to no regulation on how herbal supplements are made and processed. Bacterial contaminants could be from other sources besides the plant, as consumers do not always know what other ingredients are in the supplement capsules. The manufacturers of the supplements studied did list other ingredients contained in the supplements, such as rice powder (potentially a source of the *Oryza* sequences), rice flour, and gelatin, which could be sources of the bacterial contaminants. However, the fact that manufacturers are not required to include all of the ingredients on their bottle leads one to imagine what other things are included that did not make the label.

By trying to eat healthier, some people have decided to consume a product that is unproven and unreliable. The thought that this industry is not regulated and does not provide consistent or reliable ways to determine possible health complications (Ernst 2000), ingredients (Newmaster et al. 2013), or effectiveness (Bent 2008) is mind-boggling. It is my personal opinion that herbal supplements should be regarded as a food or medicine product. The FDA

should treat this product as such and hold the manufacturers to the same standards as food and/or medical companies. This may mean more regulation of the product itself or more monitoring of the process of getting the plant from the field to the capsule. Products should be taken off the market not only if there are health complications, but also if there are any questions about their efficacy or composition. The situation as it currently stands leaves too many questions unanswered in terms of safety and reliability and something should be done to remedy them.

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