Supplementary Information

Integrative therapies in anxiety treatment with special emphasis on the gut microbiome

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Consent and ethical approval

This case adheres strictly to requirements stipulated by the University of Oklahoma Internal Review Board document SOP 406: Determination of Human Research and Protocol Development, section 1.3 (b): Case studies involving no more than two (2) separate cases, provided that the case studies are void of private identifiable information, may be initiated without prior submission to IRB.

Informed consent for participation in this study was obtained in writing from the case study client, and the client's mental health therapist. Participation in this study was completely voluntary. The client was informed that he may withdraw at any time. All expenses incurred by the client for the purposes of this study were reimbursed. The identity of the client will remain private and anonymous. Names have not be used for the sample collection, analysis, and reporting. Stool samples will not be associated in any way with their individual owner and no public report will include information that will make it possible to identify the client.

Case study protocol transcript

Baseline week

Diet:
Eat your normal foods and meals in your normal proportions, but this time you will use the test food guide to systematically measure your blood glucose response to food.

In the Test Foods tab of the excel file, you will find a list of some of the main foods that you incorporate into your normal diet. For each day of the week and for each meal, select one of those food items to prepare as normal, and take 3 blood glucose measurements for before, during, and after the meal (precise directions written below).

You will need to test these foods largely in isolation, so you will have to eat rather plain meals during the test period. The gold standard is to conduct a pre-, peri-, and post-prandial test that lasts 2 hours total. This means that you do the first test (pre) just before you eat, then after 1 hour you take the second (peri), and then at the 2 hour mark, you take the third (post).

Since the food items you are testing may not constitute a whole meal, you can have a small side with the meals, which is indicated in parentheses. Make sure these "sides" are much smaller in portion and in calories than the main test food (for carbohydrates, especially bread or crackers, no more than ~75 calories worth, or one slice of bread).

For these test meals, log each meal and portion (by weight or volume) on the log excel sheet provided. Make sure to test each of the meals at least once. You will of course have to do multiple tests per day, but only one per meal. Snacks are added to give you a chance to test more foods, as well as desserts and alcohol in case you want to try those. They are optional, but will help if you can test them. However, desserts and snacks need to be 2 hours outside of any other meal. Additionally, all test meals must be outside of 2 hours from all other meals or snacks. In general, try to adhere to your normal routine and schedule.

Blood-glucose test:
Follow the instructions for the monitor to use it. Take one measurement just before you eat and log the result. Immediately following the reading, eat your meal. Eat at your normal pace. Exactly 1 hour after the first glucose reading and the start of the meal, take your second measurement and log the result. Exactly 2 hours after the first glucose reading and the start of the meal, take your third measurement and log the result. The third reading that you take two hours after the start of the meal concludes the test. You can eat something else or even begin a new test if you wish.

Things to log:
Along with your test meals, portions, and blood glucose readings, it will be helpful if you log any physical activity (beyond basic tasks) or stressful moments that occur throughout the test week. Also log your sleep hours and quality.

Here is a stress scale to rate you stress level:

Calm		Stressed		Distressed
1	2	3	4	5

Beck Anxiety Inventory (BAI):
You will need to take the BAI at the first opportunity in session with your therapist in the first week. You can also conduct the test yourself if necessary, but professional guidance is preferred. Keep a record of

the score, either with your therapist, or in your own personal records, and submit this score along with your logs at the end of the week.

Stool sampling:
You should collect a stool sample at the beginning of the trial, and continue to do so every week at approximately the same time of day (e.g. every Monday morning). For self-collection at home, you only need a few basic supplies and your freezer.

- tongue depressors (popsicle sticks)
- bleach
- distilled water
- Ziploc freezer bags (small) or mini round plastic disposable containers with lids (4 oz)
- opaque sealable disposable container (such as used Cool Whip or yogurt tub)
- permanent marker

1) First, soak about 10 tongue depressors in bleach for about 30 seconds, then rinse off bleach with distilled water and let them dry on a rack or balanced over the lip of clean cups. Once dry, put the sterilized depressors into a Ziploc bag until use. If you have the plastic containers, then break the depressors in half so that they will fit.
2) Change the water level in your toilet so that the bowl water is very low. If possible, urinate first and flush, then defecate. Use one sterilized tongue depressor to collect a small pea or marble sized amount of stool and put directly into a new Ziploc freezer bag and then seal immediately. You can put the entire depressor and sample into the bag together.
3) Use the permanent marker to label the Ziploc bag with the date of collection and a sample number (such as “Feb 1; Sample 1”, “Feb 8; Sample 2”, etc…).
4) Put the sealed and labeled bag with the stool sample into the opaque container, seal the container and put in the freezer (your house freezer should be ~ 0 to -2 °F, or ~ -18 °C).
5) Store samples in the freezer until all samples are collected and likewise frozen until proceeding with shipping.

Test week 1

Diet: Your glucose responses did not raise any flags or show any major outliers, except for the bagel. Keep eating your normal foods in your normal proportions within your regular routine. If you can, substitute bagels for a different option, such as basic breads or English muffin.

What you will do differently is you will incorporate pro- and pre-biotic foods into all of your meals. If you can make kimchi or sauerkraut at home, try starting a batch (recipe included), and while that ferments, buy some premade sauerkraut that is actually fermented and alive, rather than pickled. It should be in the refrigerated section, and it cannot be pasteurized.

Probiotic foods (along with yogurt) – these contain active cultures: miso soup, soft cheeses like gouda, camembert, brie, or blue cheese (must be raw, unpasteurized), sour pickles, kefir (raw, unpasteurized), artisan sour doughs, and lacto-fermented condiments such as mustard, mayonnaise, fruit chutney, hot sauce, or relish.

Prebiotic foods – these encourage and feed already present gut microbiota: onion, Jerusalem artichoke, asparagus, raw carrots, dandelion greens, garlic, leek, apples, ginger, celery, jicama, radish, bananas, oatmeal, and legumes (there are many more, but this is a start).

What you will do: Eat a large mouthful, or 1 half cup (at least) of probiotic food before all of your meals. Treat it as your appetizer. You can start with using yogurt that has live cultures if that is all you have for the first day, but try to incorporate varied types of pro-biotic foods. You can eat more if you like. Also include one type of prebiotic food as a part of your meal that you eat with your meal, such as having a banana, or using dandelion greens for a salad or on your sandwich. You can include as much pre- and pro- biotic food into your diet as you want, but the minimum is 1 half cup (or large mouthful) of probiotic food at the beginning of the meal, and incorporation of at least one type of pre-biotic food during the meal.

Logbook: This time, log all of your meals and the foods you use to meet your criteria above. The level of detail you gave in the first round was sufficient. You do not need to monitor your glucose anymore if you do not want, but continue to log your sleep and make notes about feelings of anxiety, when they happen, and where you are or what you are doing. Log physical activity or energy levels, or anything else that could epitomize the mood and energy of that day.

Fecal sample: Take another one on whichever day you designated to do this, and continue to do this once a week.

Test week 2

This week you will engage in mindful exploration of your emotional experiences and physical sensations of well-being in response to your diet. This is called mindfulness and you will use it as your tool for this week, in much the same way that you used the other tools of glucose monitoring and diet interventions in the previous weeks.

You will keep your diet the same as in the last week, in which you have strategically incorporated probiotic and prebiotic foods into each meal, and you will continue to keep a food log. Therefore, the current dietary implementation that you have in place now will remain exactly the same for the next week. Continue to eat approximately half a cup of fermented food before each meal (of any form; yogurt, kefir, sauerkraut, kimchi, tempeh, miso soup, etc.), and incorporate at least one type of prebiotic food into the meal itself. These techniques can help you populate your gut with beneficial microbiota that help you digest and extract essential nutrients from your food, maintain a healthy gut ecosystem by excluding or outcompeting potential pathogenic bacteria, thus encouraging growth of a diverse community of microbiota that are more stable over time than are a few dominant opportunistic taxa.

What you will do:
You will systematically record your feelings and sensations, both emotional and physical, for each meal at set time intervals directly before eating and then 1 hour after you have finished eating. You will log these perceptions in your log book provided here for Test Week 2.

How you will do it (have pen and paper ready):

1. You will need to focus on the experience in the here and now without distractions. If you must physically move to a temporarily quieter place, then do so. You will only need about 5 minutes.

2. Now pause and close your eyes and look inward. If a distracting thought enters your mind, acknowledge the thought and let it go and again focus your attention on your emotional and physical being and sensations.

3. First acknowledge what is your perceived mood at this very moment or just a few minutes prior. Write it down.

4. Next, track your emotional state from the last three to five spontaneous thoughts you have had. What emotion do those thoughts convey? ex: irritation, anger, sadness, joy, eagerness, contentedness, anxiousness, anticipation, stressfulness, curiosity, bewilderment, confusion, laziness, unmotivated, other… Write down a few of the more prominent emotions that you identify.

5. Next, close your eyes and take a deep diaphramic breath. Starting from the top of your head, mentally track your physical being, working slowly down through your face and neck, out to your arms and fingertips, and on until you have reached your toes. Once you are finished, write down your assessment of how you feel physically, whether you have aches or pains, or trouble spots, or where you feel tension or restlessness, or whether you feel relaxed or strong or tall, or any other physical qualities that characterize your state of physical being.

Continuing this practice for the week:
If you keep a normal meal schedule of 3 meals a day, then you will conduct this mindful self-assessment 6 times a day. If you take about 5 minutes to do this per assessment, then that is only 30 minutes of your entire day that you have devoted to your inner-self. Cherish that time and view these 5 minutes as reprieves in your schedule. You may find that after a few days, this self-assessment becomes more

intuitive and easier. Allow yourself to fully experience the exercise, and if you must postpone your assessment for 5 or 10 minutes because of a temporary distraction, then go ahead and deal with the distraction so that you can come back to a peaceful and distraction-free 5 minute window.

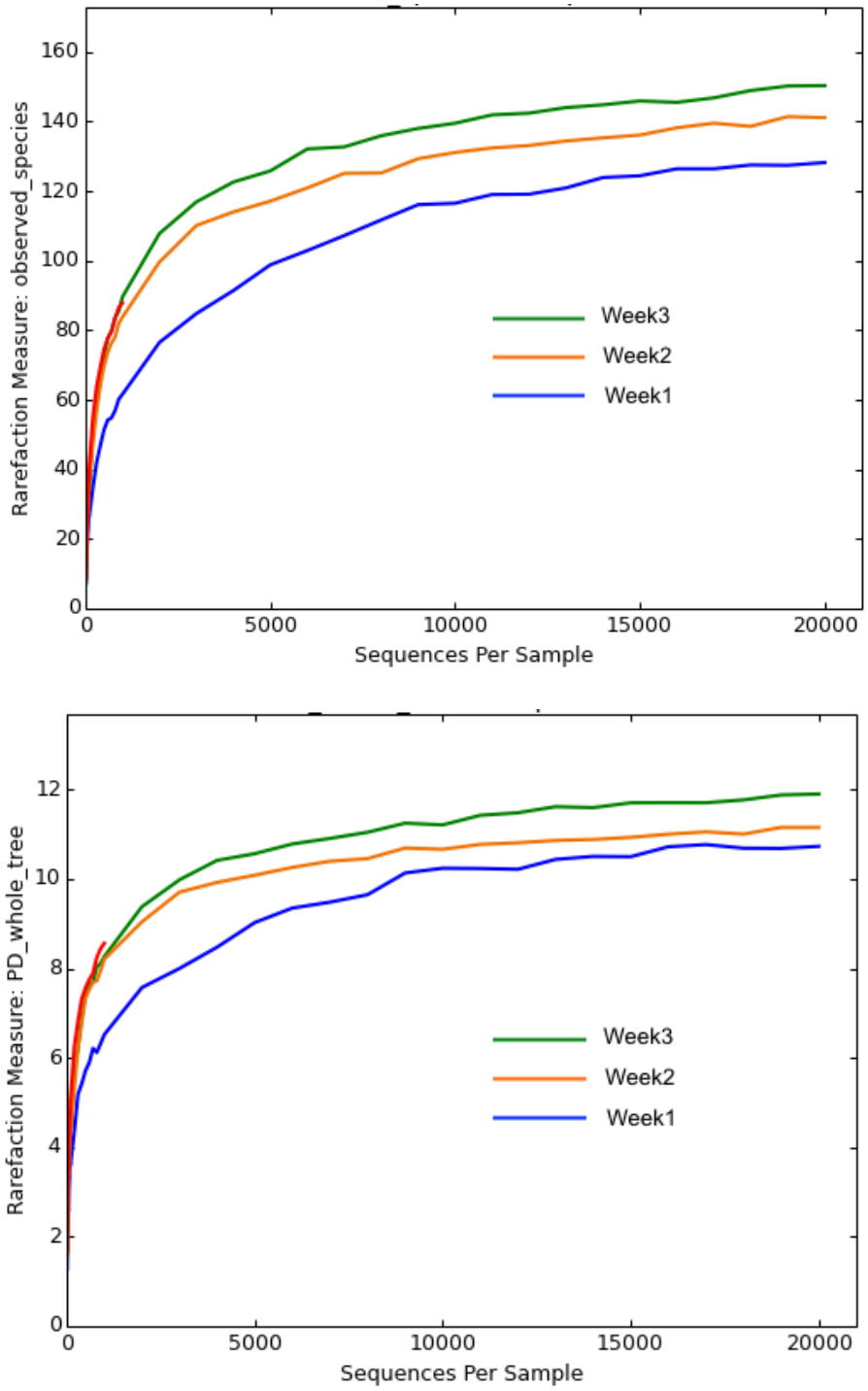
Supplementary methods

Freshly voided fecal samples were isolated and frozen immediately at -20 °C, and shipped overnight on ice. Once in the lab, samples were kept frozen at -80 °C until further handling. Extractions were conducted using the PowerSoil DNA Isolation Kit (Qiagen) and the V4 region of the 16S rRNA gene was amplified with Phusion HS using the bacterial/archaeal primers 515F/806R (initial denature at 94° for 2 mins, then 25 cycles of 94° for 15 s, 50° for 15 s, and 72° for 20 s, with final elongation at 72° for 5 mins) and sequenced on an Illumina MiSeq platform (2 x 150 bp). Paired-end reads were trimmed ($q < 30$) and assembled using PEAR [1], and OTUs were clustered at 97% identity using UPARSE [2]. Taxonomy was assigned using the Greengenes database (release 13_08) [3]. The resulting OTU table was rarefied to a depth of 15,000 reads per sample, replicated 10 times. Median OTU counts were used to generate the final OTU table, which was used for all downstream analyses. Alpha diversity metrics were calculated in QIIME [4]. Heatplot was generated in R [5] using the Made4 package and 'heatplot' function [6].

Supplementary references

1. Zhang J, Kobert K, Flouri T, Stamatakis A. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. Bioinformatics. 2014;30:614–20.
2. Edgar RC. UPARSE: Highly accurate OTU sequences from microbial amplicon reads. Nat Methods. 2013;10(10):996–8.
3. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol. 2006;72:5069–72.
4. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger J, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010;7:335–6.
5. RCoreTeam. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2013.
6. Culhane AC, Thioulouse J, Perrière G, Higgins DG. MADE4: an R package for multivariate analysis of gene expression data. Bioinformatics. 2005 Jun 1;21(11):2789–90.

Supplementary figures



Supplementary Figure 1. Alpha-diversity plots of observed species and phylogenetic diversity shown for each sample week, with incrementally increasing diversity seen across the three intervention weeks.

Food Log								
				Blood glucose readings (min)				
		Food item	Serving size	1 (t=0)	2 (t=60)	3 (t=120)	Date	Notes
Breakfast	1							
	2							
	3							
	4							
	5							
	6							
	7							
	8							
	9							
	10							
Lunch	1							
	2							
	3							
	4							
	5							
	6							
	7							
	8							
	9							
	10							
Dinner	1							
	2							
	3							
	4							
	5							
	6							
	7							
	8							
	9							
	10							
Snack	1							
	2							
	3							
	4							
	5							
	6							
	7							
	8							
	9							
Dessert/Alcohol	1							
	2							
	3							
	4							
	5							
	6							
	7							
	8							
	9							
	10							

Sleep Log				
Entry	**Date**	**Sleep hours**	**Sleep quality**	**Notes**
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				
25				

Activity and Stress Log						
Entry	Date	Time	Physical activity type	Physical activity level (1-5; 5 being maximal)	Stressful moment (1-5; 1 being calm, 5 being distressed)	Notes
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						
16						
17						
18						
19						
20						
21						
22						

Mindfulness log								
			numbers are for categorical contiguity					
			1a	1b	2a	2b	3a	3b
Entry	Date	Meal	Mood (before)	Mood (after)	Emotions (before)	Emotions (after)	Physical (before)	Physical (after)
1	23-Feb	i.e. breakfast						
2		lunch						
3		dinner						
4	24-Feb	…						
5								
6								
7								
8								
9								
10								
11								
12								
13								
14								
15								
16								
17								
18								
19								
20								
21								
22								
23								
24								
25								