



Experiment Method and Data: 010818EW

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The goal of this study was to compare the effect of the Nutribrix to the microbials NutriBrix HFGM AND NutriBrix FGM additive on the two submitted samples: The data was acquired only after 24 hour after adding Nutribrix oxygen to the samples.

1. "FGM"
2. "HFGM"

5 ml of each sample was aliquoted into 15ml conical tubes and 5 ul of Nutribrix Oxygen additive was added to each aliquot. This procedure was completed in triplicate resulting in a total of 6 treatment samples. Similarly, 5 ml of each sample was aliquoted into 15ml tubes with no additive added to be used as a control. This process was also completed in triplicate resulting in 6 control samples. The following 12 aliquots were all incubated at room temperature (~21°C) for a period of 24 hours before DNA extraction:

- 3 x FGM w/ Additive (FGM.A)
- 3 x FGM no Additive (FGM.NA)
- 3 x HFGM w/ Additive (HFGM.A)
- 3 x HFGM no Additive (HFGM.NA)

Genomic DNA was isolated from each sample using the PowerSoil® DNA Isolation Kit (Qiagen) following the manufacturer's instructions. As an alternative to the recommended 250mg of soil, each sample was centrifuged at a speed of 2000 rpm and 250ul of liquid along with the cell pellet was added to the

PowerBeads tube to undergo cell lysis. The purified DNA was eluted from the spin filter using 50uL of solution C6 and stored at -20°C until PCR amplification.

The following 16s and ITS sequencing primers were used to evaluate the microbial ecology of each sample using the Ion S5 sequencing platform:

16s: 515F GTGCCAGCMGCCGCGGTAA / 806R CCTCTCTATGGGCAGTCGGTGAT

ITS: ITS1F CTTGGTCATTTAGAGGAAGTAA / ITS2R GCTGCGTTCTTCATCGATGC

Each sample underwent a single-step 30 cycle PCR using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) were used under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds; 53°C for 40 seconds and 72°C for 1 minute; after which a final elongation step at 72°C for 5 minutes was performed. Following PCR, each amplicon product from different samples was mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA).

In addition to DNA sequencing, qPCR was performed to quantify the average number of bacteria and fungi in each sample. Each qPCR reaction was performed with 1 µL of the template DNA for each sample using TaqMan Universal PCR Master Mix (Applied Biosystems) for bacteria quantification and PowerUp SYBER® Green Master Mix (Applied Biosystems) for fungi quantification. The assay was completed using the StepOnePlus Real-Time PCR System (Applied Biosystems) in conjunction with the Bacteria2F and Bacteria2R primers for bacteria reactions and SSUfungiF and SSUfungiR primers for fungi reactions. Three replications were used for each sample, and DNA from *E. coli* was used as the bacteria standard while DNA from *Saccharomyces cerevisiae* was used as the fungi standard.

Figure 1. Average # of Bacteria and Fungi (qPCR)

	average bacteria/ul DNA		average fungi/ul DNA
HFGM_A	1162.815	HFGM_A	22127.855
HFGM_NA	1023.797	HFGM_NA	21281.567
FGM_A	117.333	FGM_A	1283.019
FGM_NA	53.879	FGM_NA	1247.031

Looking at the data from Figure 1, it is evident that the addition of the Nutribrix additive had very little effect on the number of fungi present when compared to the control sample group. Similarly, while there does appear to be a more noticeable difference in the number of bacteria detected between

samples with and without additive, that difference does not appear to be significant. The complete results of the qPCR assay can be seen in Table 3.

An analysis of variance (ANOVA) was performed evaluating the top 50 predominant genera of bacteria and fungi found in the treatment and control sample groups. Post hoc pairwise comparisons were made using Tukey's test and we evaluated whether any specific genera were significantly different between treatment and control groups. Similar to the qPCR assay, the addition of the Nutribrix additive did not produce any significant differences in either the clean or HFGM sample type with regards to the relative abundance of fungal genera (Table 2). In contrast, the ANOVA test comparing the relative abundance of bacteria detected a wide range of genera found to be significantly different between treatment groups. The results of this analysis can be seen in Table 1. The two most notable differences were found in the relative abundance of Nitrosomonas and Dechloromonas in sample types FGM and HFGM with and without additive.

Table 1. Summary of All Pairwise Comparisons with a Significant Difference found in the Mean Relative Abundance of Bacterial Genera Between Treatment Groups. *e.g. Groups belonging to category A are not significantly different from groups belonging to category AB, but are significantly different from groups belonging to categories B, C, D, BC etc.*

Groups	Means(nitrosomonas)	Statistical Categories
FGM_NA	37.748	A
FGM_A	29.017	B
HFGM_NA	24.604	C
HFGM_A	17.486	D

Groups	Means(dechloromonas)	Statistical Categories
HFGM_NA	12.372	A
HFGM_A	7.868	B
FGM_NA	1.809	C
FGM_A	0.578	D

Groups	Means(pseudomonas)	Statistical Categories
FGM_A	12.808	A
FGM_NA	7.515	B
HFGM_NA	0.354	C
HFGM_A	0.311	C

Groups	Means(oxalobacter)	Statistical Categories
FGM_A	9.254	A
FGM_NA	8.751	A
HFGM_A	0.992	B
HFGM_NA	0.914	B

Groups	Means(phenylobacterium)	Statistical Categories
FGM_A	4.445	A
HFGM_A	4.330	A
FGM_NA	2.730	B
HFGM_NA	2.490	B

Groups	Means(rhodothermus)	Statistical Categories
HFGM_A	6.489	A
HFGM_NA	6.272	A
FGM_NA	0.182	B
FGM_A	0.174	B

Groups	Means(melioribacter)	Statistical Categories
HFGM_A	4.495	A
HFGM_NA	3.547	B
FGM_A	2.529	C
FGM_NA	2.077	C

Groups	Means(rhodococcus)	Statistical Categories
FGM_A	4.306	A
FGM_NA	4.056	A
HFGM_A	0.150	B
HFGM_NA	0.031	B

Groups	Means(geobacter)	Statistical Categories
HFGM_A	3.174	A
HFGM_NA	2.604	B
FGM_A	1.219	C

FGM_NA	0.966	C
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Groups	Means(caulobacter)	Statistical Categories
HFGM_A	3.433	A
HFGM_NA	1.701	B
FGM_A	0.999	C
FGM_NA	0.624	C

Groups	Means(flavobacterium)	Statistical Categories
FGM_A	4.531	A
FGM_NA	1.800	B
HFGM_A	0.155	C
HFGM_NA	0.104	C

Groups	Means(bellilinea)	Statistical Categories
FGM_A	2.081	A
FGM_NA	1.552	AB
HFGM_A	0.955	BC
HFGM_NA	0.562	C

Groups	Means(bacteroides)	Statistical Categories
HFGM_A	2.270	A
HFGM_NA	1.832	B
FGM_A	0.280	C
FGM_NA	0.214	C

Groups	Means(burkholderia)	Statistical Categories
HFGM_A	1.448	A
HFGM_NA	1.105	B
FGM_NA	0.942	B
FGM_A	0.919	B

Groups	Means(ignavibacterium)	Statistical Categories
HFGM_A	1.479	A
HFGM_NA	1.068	B
FGM_A	0.697	C
FGM_NA	0.654	C

Groups	Means(moorella)	Statistical Categories
HFGM_A	2.130	A
HFGM_NA	1.475	A
FGM_A	0.134	B
FGM_NA	0.131	B

Groups	Means(geoalkalibacter)	Statistical Categories
HFGM_A	1.168	A
HFGM_NA	0.928	B
FGM_A	0.809	B
FGM_NA	0.739	B

Groups	Means(novosphingobium)	Statistical Categories
HFGM_NA	1.425	A
HFGM_A	1.061	AB
FGM_NA	0.549	BC
FGM_A	0.375	C

Groups	Means(heliobacterium)	Statistical Categories
FGM_NA	2.068	A
FGM_A	0.857	B
HFGM_A	0.185	C
HFGM_NA	0.149	C

Groups	Means(acidobacterium)	Statistical Categories
HFGM_A	1.478	A
HFGM_NA	1.364	A
FGM_NA	0.190	B
FGM_A	0.146	B

Groups	Means(arthrobacter)	Statistical Categories
HFGM_A	1.038	A
HFGM_NA	0.776	B
FGM_A	0.638	B
FGM_NA	0.602	B

Groups	Means(rhodanobacter)	Statistical Categories
FGM_NA	1.433	A
FGM_A	1.275	A
HFGM_NA	0.123	B
HFGM_A	0.118	B

Groups	Means(syntrophomonas)	Statistical Categories
FGM_A	1.053	A
FGM_NA	0.731	B
HFGM_A	0.612	B
HFGM_NA	0.501	B

Groups	Means(azoarcus)	Statistical Categories
HFGM_A	1.075	A
HFGM_NA	0.933	A
FGM_NA	0.410	B
FGM_A	0.386	B

Groups	Means(syntrophus)	Statistical Categories
HFGM_NA	1.139	A
HFGM_A	1.061	A
FGM_NA	0.146	B
FGM_A	0.097	B

Groups	Means(desulfococcus)	Statistical Categories
HFGM_A	1.270	A
HFGM_NA	1.024	B
FGM_A	0.036	C
FGM_NA	0.014	C

Groups	Means(pedomicrobium)	Statistical Categories
FGM_NA	0.925	A
FGM_A	0.576	B
HFGM_NA	0.516	B
HFGM_A	0.241	C

Groups	Means(sulfitobacter)	Statistical Categories
FGM_NA	1.229	A
FGM_A	0.764	B
HFGM_NA	0.041	C
HFGM_A	0.041	C

Groups	Means(desulfobacterium)	Statistical Categories
HFGM_A	0.923	A
HFGM_NA	0.863	A
FGM_NA	0.083	B
FGM_A	0.071	B

Groups	Means(rhodobacter)	Statistical Categories
HFGM_A	0.628	A
HFGM_NA	0.550	AB
FGM_A	0.401	BC
FGM_NA	0.348	C

Groups	Means(owenweeksia)	Statistical Categories
HFGM_A	0.949	A
HFGM_NA	0.771	B
FGM_NA	0.073	C
FGM_A	0.059	C

Groups	Means(bradyrhizobium)	Statistical Categories
HFGM_NA	0.771	A
HFGM_A	0.660	AB
FGM_A	0.129	B
FGM_NA	0.123	B

Groups	Means(thermoanaerobacter)	Statistical Categories
FGM_A	0.584	A
FGM_NA	0.432	AB
HFGM_A	0.392	AB
HFGM_NA	0.245	B

Groups	Means(symbiobacterium)	Statistical Categories
HFGM_A	0.797	A
HFGM_NA	0.634	A
FGM_NA	0.057	B
FGM_A	0.046	B

Groups	Means(clostridium)	Statistical Categories
HFGM_A	0.746	A
HFGM_NA	0.617	B
FGM_NA	0.086	C
FGM_A	0.067	C

Groups	Means(spirochaeta)	Statistical Categories
HFGM_NA	0.867	A
HFGM_A	0.491	B
FGM_A	0.050	C
FGM_NA	0.050	C

Groups	Means(chloroflexus)	Statistical Categories
HFGM_A	0.403	A
FGM_NA	0.363	AB
FGM_A	0.355	AB
HFGM_NA	0.307	B

Groups	Means(dehalococcoides)	Statistical Categories
HFGM_A	0.636	A
HFGM_NA	0.468	B

FGM_NA	0.183	C
FGM_A	0.132	C

Groups	Means(ochromonas)	Statistical Categories
HFGM_A	0.783	A
HFGM_NA	0.625	B
FGM_A	0.000	C
FGM_NA	0.000	C

Groups	Means(derxia)	Statistical Categories
FGM_NA	0.670	A
FGM_A	0.430	B
HFGM_A	0.131	C
HFGM NA	0.128	C

Groups	Means(sulfuritalea)	Statistical Categories
HFGM_A	0.763	A
HFGM_NA	0.538	B
FGM_A	0.013	C
FGM_NA	0.010	C

Table 2. Summary of All Pairwise Comparisons with a Significant Difference found in the Mean Relative Abundance of Fungal Genera Between Treatment Groups. *e.g. Groups belonging to category A are not significantly different from groups belonging to category AB, but are significantly different from groups belonging to categories B, C, BC etc.*

Groups	Means(phialocephala)	Statistical Categories
FGM_NA	57.705	A
FGM_A	40.896	AB
HFGM_NA	3.594	B
HFGM_A	3.293	B

Groups	Means(imaia)	Statistical Categories
FGM_NA	4.914	A
FGM_A	4.444	A
HFGM_NA	0.820	B
HFGM_A	0.197	B

Groups	Means(olpidium)	Statistical Categories
HFGM_A	1.950	A
HFGM_NA	1.581	A
FGM_A	0.020	B
FGM_NA	0.000	B

Groups	Means(scleroderma)	Statistical Categories
FGM_NA	0.576	A
FGM_A	0.493	AB
HFGM_NA	0.101	BC
HFGM_A	0.018	C

Groups	Means(burgella)	Statistical Categories
HFGM_A	0.511	A
HFGM_NA	0.391	AB
FGM_A	0.000	B
FGM_NA	0.000	B

Groups	Means(porodaedalea)	Statistical Categories
FGM_NA	0.342	A
FGM_A	0.340	A
HFGM_NA	0.039	AB
HFGM_A	0.015	B

Groups	Means(glomus)	Statistical Categories
FGM_NA	0.181	A
FGM_A	0.149	AB
HFGM_NA	0.069	B
HFGM_A	0.043	B

Table 3. qPCR Results

sample	average bacteria/ul DNA	bacteria/mL original		average fungi/ul DNA	fungi/ml original
FGM.NA.1	4.416305065	1766.522026		1388.877808	555551.1
FGM.NA.2	90.55343628	36221.37451		1169.040975	467616.4
FGM.NA.3	66.6679306	26667.17224		1183.175293	473270.1
FGM.A.1	32.57401276	13029.6051		496.8907064	198756.3
FGM.A.2	130.897522	52359.00879		1486.189535	594475.8
FGM.A.3	188.528656	75411.4624		1865.978027	746391.2
HFGM.NA.1	2018.934448	807573.7793		20888.8099	8355524
HFGM.NA.2	728.7255859	291490.2344		21643.41276	8657365
HFGM.NA.3	323.7319946	129492.7979		21312.47982	8524992
HFGM.A.1	1903.548584	761419.4336		22702.83464	9081134
HFGM.A.2	1192.176025	476870.4102		17001.93229	6800773
HFGM.A.3	392.7202148	157088.0859		26678.79688	10671519

Data taken from the averages in Figure #1

Sample:	Average Bacterial DNA	Average Fungi/ul DNA
HFGM_A	1162.815	22127.855
HFGM_NA	1023.797	21281.587
Difference Better w/NutriBrix	+139.018 or +13.58%	+843.268 or +3.96%
FGM_A	117.333	1283.019
FGM_NA	53.879	1247.031
Difference Better w/NutriBrix	+63.454 or +117.77%	+35.99 or +2.89%

Conclusion:

Statisticly significant increase where found when NutriBrix was added to NutriBrix HFGM and especially NutriBrix FGM.