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1 **Addition of insoluble fiber to isolation media allows for increased metabolite diversity of**
2 **lab-cultivable microbes derived from zebrafish gut samples**
3
4 **Short title:** Fiber increases metabolite diversity of microbes derived from zebrafish guts
5
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20 **Addition of insoluble fiber to isolation media allows for increased metabolite diversity of**
21 **lab-cultivable microbes derived from zebrafish gut samples**
22
23 **Abstract:**
24 There is a gap in measured microbial diversity when comparing genomic sequencing
25 techniques versus cultivation from environmental samples in a laboratory setting. Standardized
26 methods in artificial environments may not recapitulate the environmental conditions that native
27 microbes require for optimal growth. For example, the intestinal tract houses microbes at
28 various pH values as well as minimal oxygen and light environments. These microbes are also
29 exposed to an atypical source of carbon: dietary fiber compacted in fecal matter. To investigate
30 how the addition of insoluble fiber to isolation media could affect the cultivation of microbes from
31 zebrafish intestines, an isolate library was built and analyzed using the bioinformatics pipeline
32 IDBac. The addition of fiber led to an increase in bacterial growth and encouraged the growth of
33 species from several phyla. Furthermore, fiber addition altered the metabolism of the cultivated
34 gut-derived microbes and induced the production of unique metabolites that were not produced
35 when microbes were otherwise grown on standard isolation media. Addition of this inexpensive
36 carbon source to media supported the cultivation of a diverse community whose specialized
37 metabolite production may more closely replicate their metabolite production *in vivo*.
38
39 **Keywords:** gut microbes, *in vitro* cultivation, insoluble fiber, natural products, zebrafish,
40 metabolomics
41

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42 **Introduction:**

43 Over the past 40 years, scientists searched for alternative strategies to increase the
44 diversity of microbes isolated from environmental samples in a laboratory setting. Dubbed in
45 1932 as "the great plate count anomaly", Razumov *et al.* noted that there was a large
46 discrepancy between the viable plate count of aquatic bacteria on petri dishes compared to
47 direct microscopic counts from the same environmental habitat.[1] As more researchers reiterated
48 the lack of a diverse community *in vitro*, confirming the phenomenon, a need for modifications in
49 the cultivation process were imperative to improve isolation.

50 There are numerous variables that can be altered such as pH, salinity, temperature,
51 oxygen levels, incubation time, and carbon and nitrogen substrates to create an *in vitro*
52 environment that better represents the *in vivo* environment.[2] Microbes have evolved to live in
53 niche environments and their survival is dependent on the available simple or complex carbon
54 and nitrogen substrates. Therefore, if a laboratory medium is lacking the main organic
55 substrates that a microbe has evolved to use, this may help contribute to the plate count
56 anomaly as the microbe may fail to grow *in vitro.* One of the earliest examples in altering the
57 carbon substrate in a medium is the addition of the water-insoluble fraction of Alfalfa hay to petri
58 dishes. This addition lead to an increase in colony counts of the microbes that colonize the
59 rumen of cattle.[3] Others contain substrates such as glass or cotton-fibers as an alternative for
60 agar to evade fluctuating seaweed prices.[4,5] Altering carbon sources gained further attention in
61 the mid-2000's with reports that modifying the organic carbon substrate in media recipes altered
62 microbe growth and sporulation of environmental isolates.[6,7] This catalyzed a variety of studies
63 that altered media to increase bacterial production of pharmaceutically relevant molecular
64 classes, escalate potential biodiesel sources, as well as encourage growth of specific bacterial
65 species from animal microbiomes.[8–10] This research solidified the foundation for what is now
66 common practice to utilize several media when attempting to cultivate microbes from
67 environmental samples (Figure S1). Recent studies have revealed that utilizing different organic

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68 carbon substrates can alter bacterial metabolism, leading to the discovery of novel compounds
69 and potential biofilm inhibitors.[11,12]
70 Taking these developments into consideration, we were interested in cultivating the
71 zebrafish gut microbiome to build a library of bacteria for testing in high-throughput antimicrobial
72 and biofilm assays.[13,14] When considering the native environment of vertebrate intestines, the
73 main components of the system include the dietary nutrients entering the system for digestion
74 and the indigestible nutrients that represent the fecal matter. Therefore, for microbes living in
75 the intestinal tract, dietary fiber may serve as a large resource of nutrients and carbon. To
76 optimize bacterial cultivation and isolation from zebrafish intestines, we utilized four types of
77 media varying in nutrient density. Interestingly, the addition of organic insoluble fiber to the least
78 nutrient medium led to an increase in the number of colonies observed on isolation plates as
79 well as an increase in the diversity of colony morphologies.
80 Though the zebrafish gut microbiome varies slightly throughout development, once
81 adulthood is reached, the gut microbiome is heavily conserved and strongly colonized by γ-
82 Proteobacteria, Firmicutes, and Fusobacteria.[15,16] Using the high-throughput bioinformatics
83 pipeline IDBac,[17] we isolated and analyzed 118 individual colonies from the zebrafish gut
84 microbiome. IDBac analysis showed that our cultivation technique generated a taxonomically
85 diverse library, reflecting that of previous metagenomic studies of the zebrafish gut microbiome,
86 *vide supra*. Confirmation of a subset of the isolates with 16S rRNA gene sequencing showed
87 that out of the 118 bacterial colonies isolated, our library represents bacterial isolates from three
88 phyla. In this study we show that addition of insoluble fiber to low nutrient agar can increase the
89 number of microbes isolated from vertebrate intestines. This simple, affordable addition also
90 alters the specialized metabolite production of bacterial isolates allowing the bacteria to produce
91 different metabolites that may be more similar to those produced *in vivo* in an *in vitro*
92 environment.[18]
93

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94 **Results**

95 **Addition of insoluble fiber to low nutrient agar plates increases bacterial growth.** To
96 cultivate gut-derived microbes from zebrafish intestines, four types of agar media were used to
97 target both fast and slow growing bacteria. Plates varied in nutrient density with high vitamin
98 freshwater (HVF), NTF, SNF + fiber, and simple nutrient freshwater (SNF) decreasing in nutrient
99 density respectively (**Figure 1**).[7,19] Over 100 individual colonies were isolated over three months
100 of growth to construct a zebrafish gut microbiome library. Addition of fiber to the least nutrient
101 medium (SNF) led to a 1.7-fold increase in bacterial colony growth, surpassing the highest
102 nutrient medium (HVF). Dissection of two healthy zebrafish intestines and two plating
103 techniques in triplicate led to the isolation of 23, 38, 36, and 21 individual colonies from the
104 HVF, NTF, SNF + fiber, and SNF plates, respectively.

105 **IDBac analysis reveals that addition of insoluble fiber supports a diverse cultivation of**
106 **gut microbes from agar plates.** Library isolates were prepared for bioinformatics analysis
107 using IDBac, a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass
108 spectrometry pipeline that allows for rapid analysis of microbial proteins and specialized
109 metabolites.[17,20] The standard IDBac workflow uses a 70% aqueous formic acid solution to lyse
110 cells but we found that this procedure was not efficient at lysing the more mucoid bacterial
111 colonies isolated.[20] A trifluoroacetic acid (TFA) extraction prior to plating samples for analysis
112 was the most effective method to consistently collect viable MS fingerprint profiles for IDBac
113 analysis.[21] Measuring proteins in the 3,000 to 15,000 Da range, IDBac organized the microbial
114 isolates into a pseudo-phylogeny dendrogram based on MS fingerprint protein similarity.[17]
115 **Figure 2** highlights 82 of the 118 bacterial isolates from our library. 16S rRNA sequencing was
116 performed on a subset of isolates. In tandem, these analyses confirmed the generated library is
117 diverse containing a minimum of 15 identified species within three phyla: Actinobacteria,
118 Proteobacteria, and Firmicutes.

119 **Metabolite association network highlights changes in metabolite production with**

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120 **different carbon sources.** The IDBac pipeline also generates metabolite association networks
121 (MANs) for rapid visualization of metabolite production and allows for a simple comparison
122 between bacterial isolates grown in different conditions.[20] **Figure 3A** shows a dendrogram
123 highlighting 16 isolates that were grown on ISP2 agar, commonly used for lab cultivation, or
124 SNF + fiber agar plates.[22] In a MAN, nodes correspond to ions detected in a sample and the
125 relationships between production across strains or conditions are visualized as arrows
126 connecting nodes. The MANs of two isolates (**Figure 3B**; N2015 and S2008) imply that both
127 have unique specialized metabolite production potential and when grown on agar supplemented
128 with fiber, there is an increase in the unique specialized metabolites detected. The MAN for
129 isolate SF2016 (**Figure 3C**), which was cultivated from a SNF + Fiber plate, is a robust producer
130 of specialized metabolites. Interestingly, when comparing the two conditions, there is a larger
131 number of nodes detected when grown without fiber.

132 **Growth of gut microbes with insoluble fiber significantly increases the number of unique**
133 **metabolites produced.** High resolution liquid chromatography mass spectrometry (HR-LC-MS)
134 of bacterial extracts from library isolates, SF2016, N2105, and S2008, were analyzed using
135 XCMS, a metabolomic platform for statistical analyses of HR-LC-MS data. Through pairwise
136 comparisons, XCMS can determine features (ion and retention time pair) that are up regulated
137 in one extract versus another and visualize these differences via a cloud plot. As seen in **Figure**
138 **4A & B**, a cloud plot compares the total ion chromatograms (TIC) of two samples (i.e SF2016
139 grown on fiber agar vs. fiber agar control). Upregulated features are denoted by a circle and the
140 darker the color of the circle, the more significant the signal. The cloud plot in **Figure 4A**
141 highlights 49 features that are significantly upregulated by SF2016 when grown on fiber agar
142 versus the 15 features observed when grown on non-fiber medium ($p<0.001$; **Figure 4B**).
143 Similar cloud plots with the isolates N2015 and S2008 exhibit the increased trend with more
144 features when the isolates were grown on fiber agar ($p<0.001$; **Figures S2 & S3**). Using XCMS
145 meta-analysis, the number of significant features produced by the three isolates were organized

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146 into a Venn diagram to visualize the number of features unique to a specific isolate or shared
147 between two or more (**Figure 4C & D**). When comparing the number of features produced by
148 SF2016, N2015, and S2008 on fiber agar versus ISP2, the isolates had a 3.2, 3.3, and 1.5 fold
149 change in features from growth with insoluble fiber.

150 **Discussion:**

151 Microbial libraries for high-throughput drug discovery screening are typically generated
152 using two to six isolation media, varying in nutrient density, which select for fast or slow growing
153 bacteria from an environmental sample.[23–26] To generate a diverse library of gut-derived
154 cultivable microbes from the zebrafish intestine as well as capture the unique metabolite
155 production of this community, it is important to consider the carbon source(s) in the media. With
156 dietary fiber being a highly abundant substrate for microbes colonizing the intestines, a fourth
157 type of medium was utilized that incorporated the addition of insoluble fiber into SNF (the least
158 nutrient medium). Using four types of media varying from high nutrient density to low, HVF,
159 NTF, SNF + Fiber, and SNF respectively, 118 bacterial colonies were isolated to constitute a
160 zebrafish gut microbiome library (**Figure 1**). The addition of wet-autoclaved insoluble fiber (20
161 g/L) lead to an increase in the growth of individual bacterial colonies (**Table S1**). The highest
162 yield of bacterial isolates were collected from the average nutrient medium (NTF) however, the
163 addition of fiber to the SNF lead to nearly an identical isolate yield (**Table S1**). Of note, the
164 addition of fiber is a minimal and inexpensive addition compared to media typically used in
165 environmental isolations (**Table S2**).

166 The potential taxonomic and specialized metabolite diversity of the cultivated isolates
167 were assessed to confirm whether the isolates matched previous metagenomic sampling
168 studies.[15,16] To investigate the newly generated library, a bioinformatics platform known as
169 IDBac was used to characterize the isolates. The IDBac workflow and software were designed
170 to rapidly collect protein and specialized metabolite profiles of microbial libraries produced from
171 environmental samples utilizing a MALDI-TOF mass spectrometer.[17,20] Of the 118 isolates, 82

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172 were examined using the IDBac workflow to organize the microbial library (**Figure 2**).The
173 remaining 36 isolates were not analyzed due to either poor data quality collection during MALDI-
174 TOF MS analysis or failure to recultivate from the initial glycerol stock (**Table S1**). Based on the
175 MS protein fingerprints of the isolates, the newly constructed library represents three phyla.
176 However, a number of isolates did not group into distinct branches nor with the IDBac in-house
177 library that is built primarily from freshwater sponge-isolates,[17] cheese microbes,[27] and wild fish
178 gut-derived microbes.[24] Therefore, 16S rRNA sequencing on a subset of isolates in this library
179 was performed to confirm the identity of a subset of the isolates and provide insight towards
180 whether the cultivation efforts did support the growth of a diverse, gut microbe library (**Table**
181 **S4**). Addition of taxonomic information gathered from 16S rRNA sequencing analysis to **Figure**
182 **2** supports the groupings formed from IDBac analysis.

183 Metagenomic studies of zebrafish intestines have shown that adults are colonized by a
184 multitude of bacteria with γ-Proteobacteria, Firmicutes, and Fusobacteria as the most abundant
185 members.[15,16] 16S rRNA sequence analysis confirmed our cultivation strategy produced a
186 library that accurately represents the major components of the zebrafish gut microbiome with
187 representatives from the Firmicute, Actinobacteria, and Proteobacteria phyla (**Figure 2**).
188 Bacteria from the three phyla have been linked to the gut and overall organism health.
189 Firmicutes typically colonize the large intestines, primarily the colon, and are one of the most
190 prevalent phyla represented in the gut microbiome.[28] They have documented roles in
191 fermentation of carbohydrates as well as lipid droplet formation for energy storage.[29,30] In our
192 dendrogram we observe the same trend with a considerable number of our isolates grouped
193 into the Firmicute phylum (**Figure 2**). Of the Firmicutes isolated, *B. subtilis* was the most
194 abundant isolate in this phylum. During its lifecycle in the gut, *B. subtilis* is known to form spores
195 in half the time it takes for a laboratory isolate.[31] Thus, it is possible the increase in isolation of
196 *B. subtilis* in our library is due to the isolation of the more prolific, endogenous *B. subtilis* isolates
197 from zebrafish intestines. *B. subtilis* has also been shown to protect fish from the aquatic

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198 pathogen *Aeromonas hydrophila*, which causes inflammation and steep mortality rates.[32] With
199 *A. hydrophila* infections being a persisting problem in zebrafish research facilities, the protective
200 bioactivity of *B. subtilis* could contribute to the large representation of this species in our
201 isolation efforts.[33] Microbes responsible for maintaining homeostasis in the gut microbiome
202 system fall under the Actinobacteria phylum.[34] These maintenance microbes, which also reside
203 in the colon, make up only a small portion of the gut microbiome community even though they
204 hold a pivotal role in overall gut flora health.[28,34] Once again, our dendrogram highlights the
205 successful isolation and profiling of at least three genera of Actinobacteria such as
206 *Rhodococcus, Kocuria,*and *Mycolicibacterium* (**Figure 2**).

207 Lastly, we observe Gram-negative Proteobacteria generally found in the small intestine.
208 This population is minor with respect to the rest of the gut microbiome because many
209 Proteobacteria are opportunistic and over colonization of these microbes has been strongly
210 correlated with several human diseases.[35,36] More specifically, we isolated and profiled
211 microbes such as *Pseudomonas montelli, Aeromonas,* and *Shewanella* that have been
212 previously isolated from feces and from aquatic environments respectively.[36,37] No
213 representatives from the Fusobacteria phylum were isolated because the cultivation methods
214 used did not support the specific conditions needed to grow anaerobic bacteria. However, we
215 hypothesize that due to the observed increase in cultivation of aerobic bacterial growth in this
216 study, the same trend would hold if repeated with anaerobic conditions and would support the
217 growth of Fusobacteria.

218 Following grouping by MS protein fingerprints with IDBac, specialized metabolite
219 diversity was investigated using a metabolite association network (MAN) that corresponds to the
220 IDBac generated dendrogram. Using IDBac, a dendrogram was created grouping 16 isolates
221 grown on ISP2 or SNF + Fiber agar (**Figure 3A**). Selecting isolates N2015 and S2008 on the
222 dendrogram generated two MANs that correspond to each of the isolates (**Figure 3B**). The
223 MANs show signals found in both conditions(shared nodes) and unique signals (single nodes)

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224 that were detected when an isolate was grown with or without fiber. The MANs highlight that
225 after removing nodes associated with matrix and the two types of mediums (controls), both
226 isolates produce more unique specialized metabolites on SNF + Fiber than ISP2 (**Figure 3B**).
227 Even though neither of these strains were originally isolated from SNF + Fiber plates but rather
228 NTF and SNF media respectively, the MAN infers that metabolite production is increased by
229 exposure to insoluble fiber during cultivation. Isolate SF2016 seems to be a prolific producer of
230 unique chemistry based on the generated MAN (**Figure 3C**). Interestingly, the MAN suggests
231 that SF2016 produces more unique specialized metabolites when grown on ISP2 medium rather
232 fiber medium. In the case of SF2016, this is an example of how cultivation through the addition
233 of insoluble fiber, allowed for the isolation of a prolific metabolite producer from the gut
234 microbiome.

235 Having used IDBac as a prioritization tool, we aimed to further investigate and quantify
236 the differences in metabolite production when gut microbes are grown on medium with insoluble
237 fiber. Bacterial extracts of isolates SF2016, N2015, and S2008 grown on fiber or ISP2 agar,
238 extracted, and analyzed via HR-LC-MS. The metabolomics platform XCMS was used to analyze
239 and run statistical analysis on these extracts allowing for the selection of features that are
240 significant ($p<0.001$) and abundant (based on fold change in intensities). XCMS can perform a
241 suite of statistical analyses and output several visual representations of these statistical results
242 with one example being cloud plots.[38] **Figure 4A** shows a cloud plot comparing two TICs:
243 SF2016 grown on fiber agar (top) and a fiber agar control (bottom). When grown on fiber agar
244 SF2016 produces 41 features compared to 15 when SF2016 is grown on ISP2 ($p<0.001$;
245 **Figure 4B**). If the significance threshold is relaxed to a $p<0.01$, the number of significant
246 features between these two conditions are 174 and 49 respectively (**Figure S3**). Regardless of
247 the threshold set for significance, SF2016 consistently makes 3-fold or more features when
248 grown on medium supplemented with insoluble fiber than that without. The same trend holds
249 with isolate N2015 (**Figure S4**) with an increase in unique metabolite production when grown on

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250 fiber agar. Interestingly, though S2008 does show unique metabolite production when grown on
251 fiber agar, the cloud plot does not support that the addition of fiber lead to an increase in the
252 number of metabolites produced, as observed in previous strains (**Figure S5**).

253 Using XCMS meta analysis, which was designed to prioritize interesting metabolite
254 features from large metabolomic datasets, significant signals from each of the three isolates
255 were identified and organized into a Venn diagram.[39] Though SF2016 seems to be the most
256 prolific of the three isolates when grown with fiber (**Figure 4C**), it is clear that the same trend
257 holds in N2015 and S2008 with all three isolates showing an increase in metabolite production
258 when grown on a medium with insoluble fiber compared to ISP2 (**Figure 4C & D,** respectively).
259 Our HR-LC-MS results support that the addition of insoluble fiber to medium leads to an
260 increase in specialized metabolite production in a natural product laboratory workflow.

261 It is worth noting that there are differences in the number of observed specialized
262 metabolites between the IDBac MANs and the XCMS chromatograms. The differences in ions
263 observed can arise for a variety of reasons. Importantly, these are two different ionization
264 sources (MALDI vs electrospray ionization - ESI) and the exact mechanism by which ions are
265 produced is fundamentally different. Various functional groups on the specialized metabolites
266 themselves may favor one ionization method over the other and neither method is capable of
267 comprehensively ionizing every metabolite in a given extract. Secondly, the samples subjected
268 to the analyses are also fundamentally different. MALDI is incredibly fast (milliseconds per
269 sample) and utilizes a small amount of a microbial colony, this direct detection typically favors
270 singularly charged ions with little in-source fragmentation. Whereas ESI-LC-MS allows for a
271 concentration of the colony and surrounding agar, which likely captures a better sampling of
272 specialized metabolite production. Additionally, LC-MS analyses are time consuming with each
273 run taking at least 12 mins/colony extraction. This allows for separation of different isomers
274 which are not possible to distinguish in MALDI-TOF MS. However, ESI is known to give rise to
275 in-source fragments which may falsely increase the perceived number of specialized

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276 metabolites in a given sample. All in all, while the overall numbers of specialized metabolites are
277 different, the trends hold across these orthogonal methods. So the most prolific producer
278 observed via the IDBac generated MAN yields the highest number of features in the ESI-LC-MS
279 analysis.

280 One of the biggest challenges the field of natural products faces is the frequent
281 rediscovery of known small molecules from environmental samples.[40] Since the discovery of
282 penicillin over 75 years ago, workflows which include cultivation of select genera mainly based
283 on morphological features or 16S rRNA followed by bioassay guided fractionation have been
284 used to continue searching for new drug candidates and/or scaffolds.[41] This workflow has been
285 historically successful in elucidating novel specialized metabolites produced in high titer by
286 environmental microbes. However, advances in technologies such as bioinformatics, mass
287 spectrometry, proteomics, and synthetic biology have improved our ability to identify bioactive
288 metabolites that are produced in smaller titers. A distinct limitation of these methods however, is
289 their inability to detect or predict novel metabolites while assessing taxonomic diversity.[41]
290 Therefore, it is important to consider how changes in cultivation techniques, such as altering
291 carbon sources, can alter the isolation of specific microbes as well as the abundance and
292 variety of metabolites produced. As discussed in the introduction, work has already been
293 completed showing that altering nutrients or growth conditions can lead to increases in
294 cultivation, altered specialized metabolism, and the discovery of putative drug candidates. Thus,
295 developing new methods to propagate microbial metabolite production prior to beginning natural
296 product discovery efforts could be vital to assisting future drug discovery.

297 With the goal of constructing a taxonomically and chemically diverse zebrafish gut
298 microbe library, insoluble fiber was added to isolation medium as a carbon source. By providing
299 this solid substrate during *in vitro* growth, we observed an increase in bacterial growth to isolate
300 microbes from environmental samples. Harnessing the speed, accuracy, and sensitivity of the
301 IDBac workflow, protein and metabolite profiles of the generated library supported that the

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302 increase in cultivation observed from the addition of fiber encouraged growth of the gut
303 microbial community and suggested that providing insoluble fiber as a carbon source alters
304 metabolite production of these isolates. Metabolomic statistical analysis of three bacterial
305 isolates, SF2016, N2015, and S2008, confirmed that regardless of whether an isolate was
306 originally cultivated from a SNF + Fiber plate, addition of insoluble fiber to growth medium lead
307 to an increase in metabolite production. Further analysis is required to determine if the increase
308 in metabolite production observed represents novel metabolites that have not been cultivated *in*
309 *vitro* or if this cultivation technique allows for metabolite isolation that has traditionally only been
310 observed *in vivo.* Work is ongoing to perform biological assays and identify constituents from
311 the cultivable zebrafish gut microbes with antimicrobial and biofilm inhibition activity.

312 **Materials and Methods:**

313 *Agar plate preparation.* Agar plates were prepared by following the medium recipes detailed in
314 **Table S4**. Mediums were autoclaved for 45 minutes and cooled to 55°C prior to the addition of
315 25 mg/L of 0.22 micron sterile filtered nalidixic acid and cycloheximide (Sigma).

316 *Dissection of Zebrafish Intestines.* Animals were treated and cared for in accordance with the
317 National Institutes of Health Guide for the Care and Use of Laboratory animals. All experiments
318 were approved by the University of Illinois at Chicago Institutional Animal Care and Use
319 Committee (IACUC). Two adult *casper* zebrafish (lacking pigmentation),[42] were sacrificed at >90
320 days post fertilization with 0.1% tricaine solution for 10 minutes and bathed in 70% EtOH prior to
321 dissection. To dissect out intestines, a ventral incision was made from the front fin to the back
322 fin. If the fish was female, eggs were separated and removed to expose the intestines. Both
323 ends of the intestinal tract were cut and the tract was removed.

324 *Intestinal sample plating.* The intestines were transferred to a 1x PBS solution with three
325 sterilized glass beads. This solution was vortexed for several minutes to break up the intestinal
326 tissues. A 10-fold dilution of the intestinal solution was made before proceeding. Intestinal
327 solution was plated in triplicate using two plating techniques (1) spreading or (2) serial spotting.

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For (1) spreading, 100 µL of the intestinal sample was pipetted to the agar and spread for even distribution of the sample. For serial spotting (2), a sterilized cotton swab was dipped into the intestinal solution and spotted throughout the plate. The entire plating procedure was then repeated with the 10-fold dilution. Plates were left to grow for up to three months at room temperature (~25°C).

Gut microbe isolation. Diversity plates were frequently monitored for new growth. New colonies were transferred to ISP2 to purify to single colonies. Once the purity of the culture was confirmed, a single colony was transferred to a liquid culture of ISP2 media. This liquid culture was left to shake at 225 RPM at 25°C until the liquid culture was turbid. Once turbid, the liquid culture was used to plate the isolate on ISP2 agar and frozen stocked.

IDBac sample preparation. From the pure isolates grown on ISP2 or SNF + fiber media, a sterilized toothpick was used to transfer a portion of a bacterial colony to a sterilized microcentrifuge tube containing 5 µL of HPLC grade TFA. Samples remained in TFA for 30 minutes to facilitate cell lysis and protein extraction. Following this incubation time, DI water (20 µL) and acetonitrile (30 µL) were added to each sample, briefly vortexed, and centrifuged at 10,000 rpm for two minutes. The supernatant (1.5 µL) was mixed with 1.5 µL of α-cyano-4-hydroxycinnamic acid (CHCA) matrix for a 1:1 ratio of sample to matrix. The 1:1 sample:matrix mixture was then plated on a MALDI target plate in technical triplicate for IDBac analysis (1 µL). Measurements were performed in positive linear and reflection modes on an Autoflex Speed LRF mass spectrometer (Bruker Daltonics) equipped with a smartbeam-II laser (355 nm).

16S sequencing molecular analysis. Total genomic DNA was extracted using a commercially available DNeasy Ultraclean Microbial Kit (Qiagen) following the manufacturer's recommendations. Primer pair 27F (5’-CAGAGTTTGATCCTGGCT-3’) and 1492R (5’-AGGAGGTGATCCAGCCGCA-3’) were used to amplify the partial 16S ribosomal RNA (rRNA) gene sequence.[43] PCR conditions in a BioRad thermocycler were as follows: initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing

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354 at 60°C for 15 seconds, and extension at 72 °C for 30 seconds, and a final extension step at 72
355 °C for 2 minutes. Each amplified product was purified using QIAquick PCR Purification kit from
356 Qiagen, followed by Sanger sequencing. Data analyzed by Geneious V11.1.4 software.

357 *IDBac protein analysis*. To generate dendrograms, MALDI-TOF MS protein data was uploaded
358 to IDBac. The following settings were used to generate reported dendrogram: percent presence:
359 70, signal to noise ratio: 4, lower mass cutoff: 3,000, upper mass cutoff: 15,000, ppm tolerance:
360 1000, distance algorithm: euclidean, clustering algorithm: ward.D2, used peak
361 presence/absence as grouping criteria.

362 *IDBac Metabolite association networks*. A new dendrogram was constructed in IDBac using
363 MALDI-TOF MS data collected on the 16 isolates that were grown on both fiber and ISP2
364 medium using the same settings as previously. For MANs, isolates SF2016, N2015 and S2008
365 were selected and the following settings were applied; percent presence: 70, signal to noise
366 ratio: 4, lower mass cutoff: 200, upper mass cutoff: 2,000, ppm tolerance: 1,000, sample
367 subtraction: Matrix. The network data was downloaded as a .csv file from IDBac and
368 importanted into Gephi version 0.9.2. This was repeated with sample subtraction being changed
369 to fiber and ISP2 agar controls. In Gephi, MANs for the same isolate were compared and nodes
370 corresponding to matrix or agar controls were manually removed to generate reported figures.

371 *Metabolite extraction from bacterial isolates*. Isolates SF2016, N2105, and S2008 were plated
372 on thin (3 mL of agar in 60 mm petri dish) SNF + Fiber or ISP2 media in duplicate. Samples and
373 control plates were grown for three days at 25 °C. Entire contents of petri dish extracted with 3
374 mL of MeOH and sonicated for 30 minutes. Crude extract were used for HR-LC-MS analysis
375 and stored at -20 °C. All extractions were performed in biological replicates (N =3).

376 *HR-LC-MS data collection and XCMS analysis.* Biological extracts were pooled (1mg/mL) and
377 run on a Bruker Impact II qTOF with a C18 UPLC (Phenomenex) in two technical replicates.
378 The method for MS^1 data collection was as follows: solvent A (H20 with 0.1% formic acid) and
379 solvent B (ACN with 0.1% formic acid). 10% isocratic solvent B for 2 minutes, 10%-100%

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380 gradient of solvent B for 8 minutes, 2 minute wash of 100% solvent B and 2 minute equilibration.

381 Data was converted to .mzXML from Bruker software Data Analysis and uploaded to the online

382 metabolomics platform XCMS. Pairwise comparisons of SF2016F vs Fiber agar, SF2016I vs

383 ISP2 agar, and SF2016F vs SF2016I were run using Parameter ID: UPLC/Bruker Q-TOF pos.

384 The same analysis was run for isolates N2015 and S2008. Cloud plots were generated with the

385 following settings; p-value: 0-0.001, fold change: ≥1.5, retention time: 1-12, intensity: ≥5,000.

386 For XCMS meta analysis, pairwise jobs of each of the three isolates versus the fiber control

387 were selected and run with the following settings; fold change ≥ 1.5, max p-value ≤ 0.001, max

388 intensity ≥5,000, *m/z* tolerance 0.01, RT tolerance 60 secs. The same workflow was repeated

389 for meta analysis of each isolate with ISP2 control.

390

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511 Figure Legends

512 **Figure 1: Diversity plates of isolated gut microbes.** Using four types of agar, we observed a
513 significant increase of bacterial growth in plates supplemented with insoluble fiber. The amount
514 of growth observed in fiber supplemented plates (SNF + Fiber) was equivalent to that
515 documented in high vitamin plates (HVF).

516 **Figure 2: IDBac constructed dendrogram of cultivated bacterial isolates from the**
517 **zebrafish gut.** Our library is composed of over 80 isolates covering three phyla. To generate
518 the IDBac dendrogram the following analysis settings were used: percent presence: 70, Signal
519 to noise ratio: 4, Lower mass cutoff: 3,000, Upper mass cutoff: 15,0000, ppm tolerance: 1000.
520 Distance algorithm: euclidean, Clustering algorithm: ward.D2, used presence/absence setting
521 for dendrogram cladding.

522 **Figure 3: Comparison of metabolite production with and without fiber.** (A) IDBac
523 dendrogram of 16 isolates grown with or without fiber. (B) Metabolite association network (MAN)
524 of isolates S2008 and N2015 grown with and without fiber. (C) MAN of isolate SF2016
525 comparing the metabolites produced when grown with or without fiber. Any *m/z* nodes
526 corresponding to matrix or agar controls were subtracted prior to analysis. To generate the
527 MANs the analysis settings used were as follows: percent presence: 70, Signal to noise:4,
528 Lower mass cutoff: 200, Upper mass cutoff: 20000, ppm tolerance: 1000. Rather than displaying
529 the MAN for the entire dendrogram, specific samples were highlighted and the corresponding
530 MAN was downloaded to visualize in open graph platform Gephi (version 0.9.2) using the Yifan
531 Hu layout.[44]
532

533 **Figure 4: Gut microbe growth on fiber agar increases specialized metabolite production.**
534 HR-LC-MS data collected from metabolite extracts of library isolates SF2016, N2015, and
535 S2008 were analyzed using XCMS pairwise and meta analysis. (A) Cloud plot comparison of
536 SF2016 grown on fiber agar (top) and fiber agar control (bottom) highlighting significant
537 metabolites (dots) produced by SF2016 when grown on fiber agar. The intensity of color of a
538 circle correlates to the increased significance of the upregulated signal. (B) Cloud plot
539 comparison of SF2016 grown on ISP2 agar (top) and ISP2 agar control (bottom). The Venn
540 diagrams on the right represents the number of signals that are unique to each library isolate
541 when grown on (C) fiber agar or (D) ISP2 medium.

SNF

NTF

SNF + Fiber

HVF

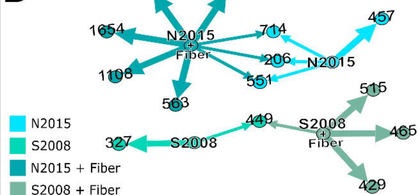
SF2010
N2017
N2015
H2007
N2010
S2008
Kocuria sp.
B. subtilis
Ensifer sp.
Ensifer sp
B. subtilis
S. hominis
SF2016
SF2015
N1020
SF2011
H1002
H2004
S1012
S1009
SF2014
H2015
N2007
Aeromonas sp.
Bosea sp.
Bosea sp.
S1001
B. subtilis
Mycolicibacteriam sp.
B. licheniformis
N1016
Bacilius sp.
N2014
Aeromonas sp.
Shewanella sp.
Shewanella sp.
B. coagulans
Aeromonas sp.
Rhodococcus sp.
Rhodococcus sp.
Rhodococcus sp.
B. coagulans
B. coagulans
P. monteilii
P. monteilii
P. monteilii
P. monteilii
B. flexus
S1006
H1007
N1003
S1005
SF1017
S1007
H1005
B. coagulans
SF1010
B. subtilis
B. subtilis
B. subtilis
B. subtilis
B. subtilis
B. subtilis
B. subtilis
B. subtilis
B. subtilis
B. subtilis
B. subtilis
B. subtilis
B. flexus
B. subtilis
B. subtilis
B. subtilis
B. subtilis
B. subtilis
B. subtilis
B. subtilis
B. subtilis
B. subtilis

Phyla
Firmicutes
Proteobacteria
Actinobacteria

15000 10000 5000 0

A

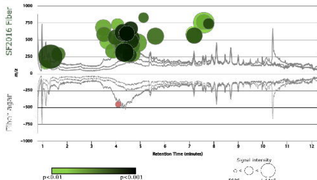
SF2010 + Fiber
SF2010
N2017 + Fiber
H1002
SF2015
N2010 + Fiber
S2008
N2015
S2008 + Fiber
N2015 + Fiber
B
SF2011 + Fiber
H2007 + Fiber
N1020 + Fiber
SF2015 + Fiber
N1020
SF2016 + Fiber
SF2016
C
SF2011
H1002 + Fiber
H2004 + Fiber
H2015 + Fiber
H2014+ Fiber
N2010
N2007
SF2014
N2007 + Fiber
SF2014 + Fiber
N2017
H2015
H2004
H2014
H2007
50 40 30 20 10 0



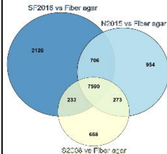
C

SF2016
SF2016 + Fiber
304 1045 1499 1097 1044 430 903 1501 1082 1513 1528 1077 1080 1096 1030 1063 1062 1492 1061 1088 338 1098 402 902 917 915 1083 666 1527 428 1046 1506 901 1477 1515 1072 918 1047 1516 1529 1031 1491 1059 1060 1076 1270 1140 693 1548 457 1965 1703 1803

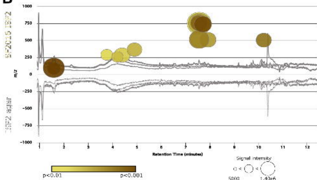
A



C



B



D