1 Metaproteomics of Gut Fungi in Gnotobiotic Mice

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11 Abstract 150 words

12 Intestinal fungi are a fundamental component of the gut microbiome and play important
13 roles in mammalian host biology. At the same time, the contribution of gut fungi to host
14 health and disease remains understudied due to their low abundance. In that respect,
15 gnotobiotic animals with defined microbial populations of reduced complexity represent a
16 well-suited model system that highlights the effects of low abundant gut fungi on host
17 physiology and other members of the microbial community. Here I present a label-free
18 quantitative metaproteomic approach for characterising simplified microbial communities in
19 gnotobiotic mice. The model allows for exploring various research questions on the role of
20 gut fungi in disease pathogenesis, microbial ecosystem maturation, or host-microbiome
21 crosstalk.

22 1. Introduction

23 The gut mycobiome defines the fungal portion of the gut microbiome, which constitutes less
24 than 0.1% of the human gut microbial ecosystem (1, 2). Despite being vastly outnumbered by
25 bacteria, fungal cells can be up to 100 times bigger in volume and contain up to 200 times
26 larger genome than most bacteria (3). The low abundant fungal species, therefore, have a
27 sizable impact on gut microbial community and intestinal homoeostasis (4, 5), as
28 documented by their roles in disease pathogenesis, including cancer (6), autoimmune (7),
29 metabolic (8), and neurological disorders (9). Characterisation of the interactions between
30 host, fungal, and other microbial cells in health and disease is thus essential for strategies
31 aiming to manipulate the gut microbiome for disease prevention and treatment.

32 From the different omics approaches, metaproteomics are uniquely positioned to describe
33 consequences of gut microbiome alterations by detecting host and microbial proteins
34 simultaneously. Faecal metaproteomes of gnotobiotic mice colonised with defined microbial
35 consortia exhibit signatures specific to the microbial status (10, 11), and provide information
36 on various cellular pathways functional in the host gut epithelium and different microbial
37 cells.

38 Here I outline methodology for describing the effects of fungal gut colonisation in gnotobiotic
39 mice (Figure 1), including practical aspects of the experimental workflow. The protocol was
40 initially applied for the evaluation of gut colonisation with six fungal strains from taxa that
41 commonly colonise the human gut (12, 13) and that have been previously linked to atopy and
42 asthma risk (11, 14). The protocol can be adapted to answer research questions on the micro-
43 ecology of specific gut fungal species or combined with different disease models in mice. In
44 general, an appropriate sample processing protocol needs to be evaluated in the context of
45 each study's aims and should consider unbiased methods for microbial protein enrichment
46 and protein extraction efficiency. Also, the availability of the genomes for all microbial strains
47 used is critical for creating matched protein databases and correct assignment of proteins.
48 Finally, the protocol will illustrate that data interpretation can be guided by several general
49 statistical analyses but that it is also, from a large part, a creative process that is unique to the
50 objectives of each specific research work.

51 2. Materials & Equipment

52 2.1 Gnotobiotic Mice

53 The experimental setup requires access to a gnotobiotic mouse facility. For
54 methodological details , see previously published protocol on germ-free mice as a
55 model for studying host-microbe interactions (15).

56 2.2 Microbial Cultivations

57 • Strains of selected gut bacterial and fungal species
58 • Selective media, e.g., fastidious anaerobic media for bacteria, yeast-mould broth
59 for fungi
60 • Anaerobic chamber
61 • 37 °C incubator
62 • Sterile culture tubes, plates and inoculation loops
63 • Sterile 1.5 ml collection tubes for faeces

64 2.3 Enrichment of microbial cells

65 • Phosphate-Buffered Saline (PBS)
66 • Tubes for gentle homogenisation and corresponding homogeniser (e.g.,
67 GentleMACS C tubes, Miltenyi Biotec)
68 • Conical centrifuge tubes of 50 ml
69 • Bench centrifuge
70

71 ## 2.4 Protein extraction

72 • Lysis buffer: 2% sodium dodecyl sulfate, 100 mM Triethylammonium bicarbonate
73 buffer, 10 mM Ethylenediaminetetraacetic acid, and 1X Complete Mini EDTA free
74 protease inhibitors, pH 8.0
75 • Tubes with lysing matrix (e.g., Fastprep lysing matrix type A,MP Biomedicals)
76 • Sample homogeniser (e.g., TissueLyser, Qiagen or Fastprep, Thermo Savant)
77 • Sonicator
78 • Spectrophotometer (for example Direct Detect® Spectrometer from Merck
79 Millipore)

80 ## 2.5 Filter Aided Sample Preparation and C18-based Peptide Clean-Up

81 • MS grade water
82 • Microcon device YM-10 filters (e.g., Merck Millipore)
83 • 100 mM ammonium bicarbonate (NH_4HCO_3 - ABC) - 79 mg ABC into 100 ml MS
84 grade water.
85 • 10 mM dithiothreitol (DTT)- 1.54 mg DTT into 1mL 100mM ABC
86 • Urea buffer (8 M urea, 10 mM HEPES, pH 8.0)
87 • 0.05 M iodoacetamide
88 • 40 mM ABC - dilute from 100 mM ABC
89 • Proteomics grade Trypsin in 40 mM ABC buffer
90 • C18 solid-phase extraction cartridges (e.g., SepPak Waters)
91 • Methanol
92 • Acetonitrile (ACN) 2%
93 • Formic acid (FA) 0.1% and 100%

94 - Elution buffer (80% ACN, 0.1% FA)
95 - Vacuum concentrator
96 - Eppendorf tubes (0.5, 1 and 2 ml)
97 - Low-bind protein tubes
98 - Peptide colorimetric assay (e.g., Pierce™ Quantitative Colorimetric Peptide Assay
99 (Thermo Fisher Scientific, Waltham, Massachusetts, USA

100 2.6 LC-MS/MS and Data Analysis

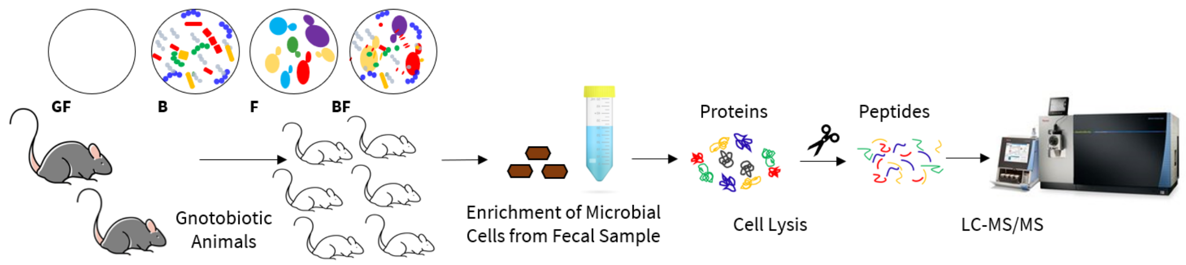
101 - Example of LC-MS/MS system (Thermo Scientific): EASY nLC 1200 connected to an
102 Orbitrap Fusion Lumos Tribrid mass spectrometer with an EASY-Spray column
103 - MaxQuant software package (16) or other freely available proteomic software
104 - Protein sequence database in FASTA format matching the studied microbial strains
105 (*e.g.*, downloaded from NCBI or UniProtKB).
106 - Spreadsheet editor and freely available Perseus software or various statistical R
107 scripts/ package (17).
108

109 ## 3. Methods

110 ### 3.1 Gnotobiotic mice

111 1. Germ-free (GF) mice need to be housed at a dedicated gnotobiotic mouse facility, and
112 all the animal work must follow animal protocols approved by a corresponding
113 institution.
114 2. Selected microbial species are grown under anaerobic conditions in their respective
115 selective media until a stationary phase.
116 3. To generate gnotobiotic mice, female adult germ-free mice are orally gavaged twice,
117 three days apart, with 100 μl of selected microorganisms (*e.g.*, strains of fungal
118 species of interest) or microbial consortia (Fig. 1, Note 1).
119 4. After the second gavage, mice are paired with gem-free males for mating in a 2:1
120 female:male ratio per cage. Two breeding pairs are used for each experimental group
121 (*i.e.*, colonisation condition) producing on average 6 offspring (±3). (Note 2)
122 5. To ensure microbial colonisation with the desired consortia in the offspring, the
123 corresponding inoculum should be further spread on the dams abdominal and nipple
124 regions on days 3 and 5 after birth. Microbial engraftment can be confirmed by Sanger
125 sequencing of DNA isolated from faecal samples.
126 6. Mice are often kept at a maximum of five animals per cage and housed inside
127 gnotobiotic isolators. Standard conditions include a 12-h light/12-h dark cycle, 40%
128 relative humidity, 22–25 °C, and *ad libitum* access to sterile food and water.

129



130 Figure 1 A gnotobiotic model for characterisation of fungal gut colonisation by label-free

131 quantitative metaproteomics. Abbreviations of potential colonisation conditions indicated in

132 circles: B, bacteria only; BF, bacteria-fungi; F, fungi only; GF, germ-free.

133 ### 3.2 Optional - Enrichment of microbial cells from faecal samples (Note 3)

134 1. Pooled faecal samples of ~ 300 mg are collected from co-housed gnotobiotic mice of
135 the same treatment group, at chosen time points and immediately stored at −80°C
136 until use.
137 2. After thawing at 4°C, samples are subjected to differential centrifugation to enrich for
138 microbial cells, according to previously described methodology (18).
139 3. Each sample is resuspended in 4 ml of Phosphate-Buffered Saline (PBS), gently
140 homogenised, and subjected to low-speed centrifugation at 20 × g for 5 min to
141 eliminate gross particulate material.
142 4. The supernatant is transferred to 50 ml conical centrifuge tube and kept at 4°C,
143 whereas the pellet is resuspended in PBS.
144 5. The washing step is repeated until the supernatant appears translucent (5-7 times).
145 6. The collected supernatant is centrifuged at 3,200 × g for 1 h.
146 7. The resulting pellet is subjected to cell lysis and protein digestion described below
147 (Note 4).

148 ### 3.3 Protein Extraction

149 1. Faecal microbiota samples are resuspended in lysis buffer in 1:4 w/v ratio and transfer
150 into a 2 ml screw-cap tube containing a garnet matrix and a ceramic sphere.
151 2. Cells are mechanically disrupted by bead-beating in a tissue lyser with settings
152 relevant for stool or soil samples.
153 3. To ensure lysis, the samples can be further incubated at -80°C for 10 min and at 95°C
154 for 10 min, followed by centrifugation for 30 min at 18,000 x g, 4°C.

155 4. To disrupt released cellular DNA that would interfere with downstream protein
156 quantification, the supernatants are sonicated 3 x for 10s with 20s resting intervals on
157 ice.
158 5. Sonicated samples ae centrifuged at 18,000 x g, 4°C, for 10 min, the supernatants
159 collected, and protein concentration is measured by using a spectrophotometer.

160 3.4 Sample preparation for the proteomic analysis

161 The cell lysates of faecal microbiota-enriched samples are processed according to Filter-
162 Aided Sample Preparation protocol (18, 19).

163 1. Cell lysates containing 500 μg of total protein are incubated with 10 mM DTT in 100
164 mM Ammonium Bicarbonate (ABC) at the solution to total protein ratio (v/w) 1:10 for
165 45 min at 56°C without shaking.
166 2. YM-30 Microcon filter units are condition by adding 100 μl of urea buffer and
167 centrifuge at 14,000 × *g* for 5 min.
168 3. The denatured protein samples from step 1. are mixed with 200 μl urea buffer in the
169 filter units and centrifuged at 10,000 × g for 15 min.
170 4. After discarding the eluate, the filtration units are washed once with 200 μl urea buffer
171 (10,000 × g, 15 min).
172 5. The filtrate is discarded and 100 μl of 0.05 M iodoacetamide added to each sample.
173 The samples are then mixed at 600 rpm for 1 min in a thermo-mixer, and incubated
174 without mixing in the dark for 20 min,
175 6. The filter units are washed with 100 μl urea buffer three time, followed by three
176 washes with 100 μl 50 mM ABC (10,000 × g, 10 min).

177 7. Proteins are digested on the filter unit with trypsin in 40 mM ABC (enzyme to protein
178 ratio (v/w) of 1:100) at 37°C for 18 h.
179 8. The released peptides are collected by adding 50 µl of MS grade water followed by
180 centrifugation at 14,000 x *g* for 15 min. Repeat this step twice.
181 9. The resulting peptide mixtures are cleaned up by using C18 solid-phase extraction
182 cartridges.
183 10. The collected samples are dried at 30°C in a vacuum concentrator and store at -80°C
184 until further analyses.
185 11. Prior to LC-MS/MS analysis, resuspend the peptide samples by following instructions
186 from chosen proteomic facility, for example by adding 1 µl of 100% formic acid and 19
187 µl of 2% ACN or only in 1% formic acid.
188 12. An aliquot of the tryptic digests can be used to determine the concentration of the
189 peptide mixtures by using colorimetric peptide assay kit.

190 ### 3.5 LC-MS/MS

191 The MS/MS analysis is typically carried out at a dedicated proteomic facility by personnel
192 operating the instruments. An example of a potential LC-MS/MS run setup is on an Orbitrap
193 Fusion Lumos Tribrid mass spectrometer operated with Xcalibur software and coupled to
194 Easy- nanoflow liquid chromatography 1200 system (Thermo Fisher Scientific). Below is an
195 example of an LC-MS/MS analytical run; however, each proteomic facility will have own
196 protocols matching their instrumentation and specific quality controls (Note 5).

197 1. Load 2 µg tryptic peptide sample onto a pre-concentration column (for example,
198 Acclaim PepMap 100, 2 cm ×75 µm i.d. nanoViper column, packed with 3 µm C18

199 beads) at a flow rate of 2 μl/min of solvent A (0.1% formic acid and 3% acetonitrile in
200 LC-MS grade water).
201 2. Separate the peptides by a biphasic acetonitrile gradient (flow rate of 300 nl/min) on a
202 C18 analytical column (e.g., 75 μm x 50 cm; PepMap RSLC C18). Specifically, apply
203 solvent B (0.1% formic acid in 80% LC-MS grade acetonitrile) during a 120 min
204 gradient from 5 to 40% (5% to 28% in 105 min followed by an increase to 40% B in 15
205 min) at a flow rate of 0.3 μL/min to elute the peptides.
206 3. The separated peptides are directly electrosprayed using 2.1 kV voltage into the ion
207 transfer tube (300°C) of the MS instrument operating in positive mode.
208 4. Operate the mass spectrometer in data-dependent acquisition mode to automatically
209 switch between MS and MS/MS acquisition.
210 5. Full scans are acquired at 120,000 Full-Width at Half Max resolution to detect the
211 precursor ions having m/z between 375 and 1,575 (Scan Range) and a +2 to +7 charge
212 (automatic gain control at 4 x 10^5 and maximum injection time 50 ms).
213 6. Precursor selection: The Orbitrap is operated using the top speed mode with a 3 sec
214 cycle time. The most intense precursor ions presenting a peptidic isotopic profile and
215 having an intensity threshold of at least 5,000 are isolated using the quadrupole and
216 fragmented by higher-energy collisional dissociation (HCD, 30% collision energy) in
217 the ion routing multipole.
218 7. The fragment ions (MS2) are then analysed in the ion trap at a rapid scan rate
219 (automatic gain control at 1 x 10^4 and maximum injection time 35 ms).
220 8. Target ions already selected for MS/MS are dynamically excluded for 45s to avoid the
221 acquisition of the same precursor ion having a similar m/z (plus or minus 10 ppm).

222 ### 3.6 Protein identification and quantitation

223 1. The acquired MS raw data can be processed by using an open-source proteomic
224 software such as MaxQuant (16) with default settings and additional options such as
225 Label-Free Quantification (LFQ) and match between different sample runs. By using
226 the LFQ option, the software will derive normalised spectral protein intensities by the
227 MaxLFQ algorithm that applies protein-specific correction coefficients on the whole
228 dataset (20).
229 2. Upload into MaxQuant matching protein databases for the used microbial strains:
230 genome-derived proteomes for specific species if these are available, or non-
231 redundant protein databases for bacterial or fungal species from the Universal
232 Protein Knowledgebase.
233 3. Perform the MS searches by analysing all raw MS data together ; depending on the
234 data size, the search might take from several hours to several days.
235 4. The MaxQuant output data ('proteingroups.txt') can be analysed with the Perseus
236 module (17), starting with filtering of protein identifications as follows: first are
237 removed proteins marked as “reverse”, “only identified by site”, and “potential
238 contaminant”. Next, only proteins identified in at least two biological replicates
239 should be considered as confident identifications.
240 5. To identify false-positive identifications of microbial proteins in mice groups not
241 colonised with bacteria and/or fungi, the protein identification type should be
242 checked, and proteins identified by the “match between runs” algorithm and not
243 directly identified by MS, filtered out. Also, proteins identified only by a single peptide
244 and not identified by several unique peptides represent low confidence identification

245 and should be removed. Any other remaining proteins, whose origins are not
246 consistent with the type of microbial colonisation of a specific mice group should be
247 checked at the peptide level, to confirm whether these are valid identifications or not.
248 6. For protein quantification, only proteins with LFQ intensities in at least two biological
249 replicates and identified by a minimum of two unique peptides should be considered.

250 ### 3.7 Data analyses

251 1. Before any analyses, the proteins LFQ intensities should be log2-transformed. To
252 correct for differences in the sample amounts injected into LC-MS/MS, the relative
253 protein amounts can be normalised by dividing each protein LFQ intensity by the
254 median intensity for all proteins in a given replicate (21).
255 2. To assess the biological variability of each experimental group, the Pearson
256 correlation coefficients based on the protein intensities of each sample can be
257 calculated in Perseus.
258 3. The statistical analyses can be performed on specific groups with defined microbial
259 status. Only relevant mice groups have to be used in the statistical analyses of
260 bacterial or fungal proteins, *e.g.*, only those mice groups colonised with fungi will be
261 used to analyse fungal proteins.
262 4. To identify proteins with levels that differ among the strains, analysis of variance
263 (ANOVA) can be used to compare the global mean level of each protein against its
264 corresponding amount in each condition. Standard settings for the ANOVA test in
265 Perseus include FDR calculations performed by a permutation-based procedure with
266 250 randomisations and a cut-off of 5%. To determine the exact pairwise differences

267 in protein levels, Tukey's honestly significant difference (THSD) should be performed
268 on ANOVA-defined significant hits.

269 5. Perseus also allows for additional uni- and multivariate analyses such as principal
270 component, multivolcano, or hierarchical clustering analyses.

271 6. The functional protein classification is usually be done using The Database for
272 Annotation, Visualisation and Integrated Discovery (22) and Gene Ontology
273 enrichment (23).

274 7. To ensure open and fair science, the mass spectrometry proteomics data should be
275 deposited to a repository database (*e.g*, ProteomeXchange).

276

277 Notes

278 1. A well-described bacterial consortium of mouse-derived strains that are persistent,
279 inheritable and elicit an immune response in mice similar to a complex microbiota is
280 the Oligo-MM12 consortium (24, 25). The method for mice colonisation with Oligo-
281 MM12 includes preparation of the gavage inoculum under anaerobic conditions by
282 mixing 100 μl of 2-day-old microbial cultures of each species.
283 2. For evaluation of physiological changes induced by microbial colonisation, each
284 animal constitutes an individual biological replica in the gnotobiotic experiment.
285 However, for metaproteomics sampling, pooled stool sample collected from animals
286 housed in the same cage, and having the same microbial status, is often necessary
287 due to limited stool material that each animal can produce, and relatively large
288 volume of stool (at minimum 300mg) needed for metaproteomic sample processing.
289 The LC-MS/MS analysis can then be performed on replicates of pooled faecal samples
290 collected from mice that underwent the same microbial colonisation and were
291 housed in the same cage and gnotobiotic isolator.
292 3. Different enrichment methods, such as strategies based on double filtering (26) and
293 differential centrifugation (18), have been applied to concentrate microbial cells from
294 stool samples and shown to improve the overall depth of faecal metaproteome
295 measurement. However, the differential centrifugation step was later shown to cause
296 non-specific removal of microbial cells and proteins (27). Stool without a pre-
297 treatment thus might provide an unbiased representation of the microbial proteins
298 but lead to a lower number of proteins identified, decreased microbial diversity, and
299 overrepresentation of the nonmicrobial components (host and food). A critical

300 evaluation is therefore needed when selecting the appropriate stool sample
301 processing protocol in the context of each metaproteomic study, depending on the
302 research aim.

303 4. A quality control step comprising of microscopic examination of Gram-stained
304 fractions of the pellet should be included to confirm bacterial and fungal cell
305 extraction.

306 5. For optimal performance, the LC-MS/MS instrumentation should be calibrated before
307 each sample batch, for example, by injecting a commercial protein digest to control
308 the performance of the LC and the mass spectrometer before the samples are
309 acquired.

310 6. The LFQ approach of Maxquant is based on accurate determination of spectrometric
311 signal intensities (extracted ion chromatograms - XICs) of peptides and relies on
312 measurements of the three-dimensional space of peptide ion intensity, m/z, and
313 chromatographic elution time. However, for proteins at low abundance, XICs are often
314 contaminated by nearby signals, and although a protein can still be identified, it
315 might not be quantified because of low-quality data.

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