

1 Deconstructing higher-order interactions in the microbiota: A theoretical 2 examination 3

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13 Abstract

- 14 1. The animal gut is a complex ecosystem containing many interacting species. A major
15 objective of microbiota research is to identify the scale at which gut taxa shape hosts.
16 However, most studies focus solely on pairwise interactions and ignore higher-order
17 interactions involving three or more component taxa. Higher-order interactions
18 represent non-additive effects that cannot be predicted from first-order or pairwise
19 interactions.
20 2. Possible reasons as to why studies of higher higher-order interactions have been
21 scarce is that many host-associated systems are experimentally intractable, gut
22 microbiota are prohibitively species rich, and the influence of any given taxon on
23 hosts is often context-dependent. Furthermore, quantifying emergent effects that
24 represent higher-order interactions that are not simply the result of lower-order
25 interactions, present a combinatorial challenge for which there are few well-
26 developed statistical approaches in host-microbiota studies.
27 3. In this perspective, our goal is to quantify the existence of emerging higher-order
28 effects and characterize their prevalence in the microbiota. To do so, we adapt a
29 method from evolutionary genetics used to quantify epistatic effects between
30 mutations and use it to quantify the effects of higher-order microbial interactions on
31 host infection risk.
32 4. We illustrate this approach by applying it to an *in silico* dataset generated to resemble
33 a population of hosts with gut-associated microbial communities. We assign each host
34 a pathogen load, and then determine how emergent interactions between gut taxa
35 influence this host trait.
36 5. We find that the effect of higher-order interactions generally increases in magnitude
37 with the number of species in the gut community. Based on the average magnitude of
38 interaction for each order, we find that 9th order interactions have the largest non-
39 linear effect on determining host infection risk.
40 6. Our approach illustrates how incorporating the effects of higher-order interactions
41 among gut microbiota can be essential for understanding their effects on host
42 infection risk. We conclude that insofar as higher-order interactions between taxa may
43 profoundly shape important organismal phenotypes (such as susceptibility to
44 infection), that they deserve greater attention in microbiome studies.

45

46

47 **Introduction**

48 Animal guts contain complex microbial communities whose structure and function
49 depend upon the interactions among microbes and the host. Gut microbiota serve as
50 key actors in host health, impacting development, metabolism, and pathogen
51 susceptibility (Brugman et al., 2018). The development of microbe-free (also known as
52 germ-free) model hosts has made it possible to experimentally study how the
53 microbiota influences host susceptibility to infection (Goodman et al., 2011; Ridaura et
54 al., 2013). However, most studies rely on correlations between the relative abundances
55 of individual bacterial taxa and host infection risk (e.g. pathogen load), ignoring the
56 potential influence of higher-order interactions between taxa within the community.
57 The field of complex systems is increasingly interested in understanding the emergent
58 properties of higher-order interactions between objects (Lambiotte, Rosvall, & Scholtes,
59 2019a). Relatedly, a long-standing issue in ecology is to capture the vast diversity of
60 multispecies species interactions—the unpredictable effects that arise when multiple
61 species are present in an ecosystem (Hutchinson 1962). For example, the order of
62 arrival of species into an ecosystem, and other factors (deterministic or stochastic in
63 nature) can dictate species composition and the overall behavior of the system
64 (Saavedra et al., 2017; Uricchio, Daws, Spear, & Mordecai, 2019). This problem has more
65 recently become the object of inquiry in communities of microbes (Enke et al., 2019;
66 Mickalide & Kuehn, 2019; Sanchez-Gorostiaga, Baji •, Osborne, Poyatos, & Sanchez,

67 2018). Many ecological studies involving complex network structures typically focus on
68 pair-wise interactions and tend to ignore higher-order effects among three or more
69 components (Kareiva, 1994; Levine, Bascompte, Adler, & Allesina, 2017; Mayfield &
70 Stouffer, 2017). For example, in a system with two interacting microbes—*A* and *B*—the
71 addition of a third microbe *C* may alter the pairwise interaction between *A* and *B* in a
72 non-linear or non-intuitive fashion. This would constitute an emergent higher-order
73 interaction between *A*, *B* and *C*. This is in contrast to a scenario where the microbe *C*
74 interacts with either *A* or *B* in isolation, which constitute pairwise interactions with their
75 own interaction effects. Therefore, quantifying emergent higher-order effects between
76 microbial taxa is necessary to fully capture the structure and dynamics of biological
77 systems.

78
79 Higher-order interactions have recently been the object of study in the realm of genetics,
80 where they are discussed in light of epistasis, or non-linear interactions between genes
81 and mutations (Mackay & Moore, 2014; Weinreich, Lan, Jaffe, & Heckendorn, 2018a;
82 Weinreich, Lan, Wylie, & Heckendorn, 2013). A useful non-technical definition of
83 epistasis is the “surprise at the phenotype when mutations are combined, given the
84 constituent mutations’ individual effects (Weinreich, Lan, Jaffe, & Heckendorn, 2018b).
85 This effectively captures what makes epistasis a provocative concept: the notion that
86 interacting objects or parcels can have effects that are non-additive. In particular,
87 higher-order epistasis is of interest, as it comprises all of the complexity and challenges

88 of understanding and studying higher-order interactions in other systems (Lambiotte et
89 al., 2019a).

90

91 Higher-order epistasis can have powerful effects on organismal phenotypes, which has
92 complicated the genotype-phenotype mapping problem in genetics (Sackton & Hartl,
93 2016). To study higher-order epistasis in model organisms, molecular biologists
94 engineer genes and mutations of interest in all possible permutations, a method labeled
95 the “combinatorial approach.” (Weinreich et al., 2018b, 2013). Other studies resolve
96 higher-order epistasis through more advanced statistical methods (Guerrero, Scarpino,
97 Rodrigues, Hartl, & Ogbunugafor, 2019; Otwinowski, McCandlish, & Plotkin, 2018;
98 Poelwijk, Krishna, & Ranganathan, 2016; Sailer & Harms, 2017).

99

100 Insect gut microbiota have been used as model systems to study the formation and
101 assembly of microbial communities. Insect guts harbor relatively fewer microbial
102 species, as compared to higher eukaryote hosts, with restricted core-members that can
103 be grown axenically and manipulated genetically (Zheng, Steele, Leonard, Motta, &
104 Moran, 2018). The protective function of microbes against invading pathogens have
105 been studied across a range of insect hosts. For example, previous studies with bees
106 found that core gut species were associated with increased host health, while non-core
107 taxa were associated with decreased host health and increased pathogen infection
108 (Cariveau, Elijah Powell, Koch, Winfree, & Moran, 2014; Koch & Schmid-Hempel, 2011;
109 Raymann & Moran, 2018). However, other studies have also shown that pathogens alter

110 the gut microbiota and facilitate gut infections (Abraham et al., 2017; Wei et al., 2017).

111 Although many studies have shown correlations between core species and host traits,

112 the extent to which individual versus species interactions facilitate or resist gut

113 infections remains understudied.

114

115 Not unlike genomes, societies or neural circuits, insect gut microbiomes are complex

116 systems defined by the interaction between individual parcels (component taxa in the

117 microbiota). Consequently, we might predict that higher-order interactions between

118 taxa in the microbiota might underlie microbiota-associated organismal phenotypes,

119 such as susceptibility to infection. Recent work by Gould et al. 2018 found that higher-

120 order interactions in the gut microbiota impact lifespan, fecundity, development time,

121 and bacterial composition of *Drosophila* sp. With a gut community composed of 5 core

122 taxa, they found that three-way, four-way, and five-way interactions accounted for 13-

123 44% of all possible cases depending on the host trait. Yet, lower-order interactions (2-

124 pairs) still accounted for at least half of all the observed phenotypes in the system

125 (Gould et al., 2018).

126

127 Studies like Gould et al. 2018 provide an example of how higher-order interactions can

128 be measured and suggest that they might be relevant for understanding how taxa

129 influence certain phenotypes. But while the importance of diversity and host

130 interactions is clear, no studies have attempted to specifically disentangle effects

131 beyond four or five-way interactions. One major barrier to more of these studies is the

132 paucity (or non-existence) of the datasets structured like those in an evolutionary
133 genetics framework, such that existing statistical methods might be used to resolve
134 interactions. (Tekin, Savage, & Yeh, 2017; Wood, Nishida, Sontag, & Cluzel, 2012). For
135 example, the problem of constructing a set of insects that each carry a different
136 combination of constituent taxa of interest grows exponentially with the number of
137 taxa. And (perhaps) unlike genetics, constructing a different insect with a different set
138 of bacterial taxa (corresponding with the possible combinations of taxa) is a non-trivial
139 technical challenge. Nonetheless, the use of combinatorial complete datasets—insects
140 containing all combinations of taxa— to explore higher-order interactions (beyond a
141 single taxon or pairwise interactions) could help to inform how taxa interact in framing
142 organismal phenotypes.

143

144 In this commentary, we propose a theoretical examination of higher order interactions
145 in the gut microbiome. Specifically, we employ the Walsh-Hadamard transform
146 (WHT), a mathematical regime that has been used to demonstrate how higher-order
147 interactions between mutations influence fitness or other organismal traits (Poelwijk et
148 al., 2016; Weinreich et al., 2013), to explore how higher-order interactions among gut
149 taxa can influence host infection risk. We use it to quantify higher-order interactions in
150 an *in silico* dataset resembling the type of data that can be empirically—that can be
151 developed in the future—collected from insect guts. We introduce this approach with
152 the hope that it may eventually be applied to a tractable experimental system for real-

153 world validation, and believe that insect systems are among the most promising
154 empirical systems.

155

156 **Methods**

157 The Walsh-Hadamard Transform allows one to quantify the eminence of interaction
158 effects of different order in a system of potentially-interacting objects or parcels. It
159 yields a Walsh coefficient, which communicates the magnitude and sign of how a
160 particular order interaction influences an output of interest. It implements phenotypic
161 values in the form of a vector, before reformatting it into a Hadamard matrix (and is
162 then scaled by a diagonal matrix). The output is a collection of coefficients which
163 measure the degree to which the map is linear, or second order, third, and so forth. We
164 provide a brief primer on the method, and refer readers to two published
165 manuscripts—Poelwijk et al. (2016) and Weinreich et. al. (2013)—that outline and apply
166 the method in good detail. Also see the Supplementary Information for a brief primer.

167

168 The Walsh-Hadamard Transform relies on the existence of combinatorial data sets,
169 where the objects for which we are interested in understanding the interactions between
170 (taxa in this study) are constructed in all possible combinations. Another limitation of
171 the WHT is that it can only accommodate two variants per site, that is, two states per
172 actor. In the case of taxa, we can think of this in terms of the presence/absence of a
173 certain taxon, and we can encode this in terms of 0 (absence) or 1 (presence). For each
174 hypothetical insect with a different presence/absence combination, we have a

175 corresponding phenotypic measurement (e.g. parasite load). For example, if we wanted
176 to measure the higher-order interactions between 4 taxa within an insect with regards to
177 their role in parasite load (as a model phenotype), we would need $2^L = 16$ individual
178 measurements (insects in this case), with L corresponding to the number of different
179 taxa whose effects we were interested in disentangling. We can encode this
180 combination of 4 taxa in bit string notation (see Figure 1).

181
182 Each site (0 or 1) in the string corresponds to the presence or absence of a given taxa in a
183 given insect. This notation allows us to keep a mental picture of which taxa are in which
184 insect for which we have a phenotypic measurement and can be used to construct a
185 vector of values. For example, the string 1010 corresponds to an insect with the pattern
186 of present (1), absent (0), present (1), absent (0). The full data set includes a vector of
187 phenotypic values for all possible combinations of taxa—0000, 0001, 0010, 0100, 1000,
188 0011, 0101, 0110, 1001, 1010, 1100, 0111, 1101, 1011, 1110, 1111. Note that these can be
189 divided into different classes based on the “order” of the interaction. Order corresponds
190 to the number of interacting actors. “Zeroth order” would correspond to the 0000
191 variant. This would translate to an insect that has none of the insect taxa present. There
192 are 4, 1st order interactions (0001, 0010, 0100, 1000), 6, 2nd order (or pairwise) interactions
193 (0011, 0101, 0110, 1001, 1010, 1100), 4, third-order interactions (0111, 1101, 1011, 1110),
194 and 1 fourth order interaction (1111). The WHT will quantify

195 This vector of phenotypic values for the 16 will be multiplied by a (16 x 16) square
196 matrix, which is the product of a diagonal matrix V and a Hadamard matrix H . These
197 matrices are defined recursively by:

$$V_{n+1} = \begin{pmatrix} \frac{1}{2} & 0 \\ 0 & -V_n \end{pmatrix}, V_0 = 1 \quad [1]$$

198
199

$$H_{n+1} = \begin{pmatrix} H_n & H_n \\ H_n & -H_n \end{pmatrix}, V_0 = 1 \quad [2]$$

200
201

202 n is the number of loci ($n = 4$ in this hypothetical example). This matrix multiplication
203 gives an output:

$$\gamma = VIIx$$

204
205

206 Where V and H are the matrices described in [1] and [2] above, and y is the Walsh
207 coefficient, the measure of the interaction between parcels of information in a string.
208 Using this, we compute y values for every possible interaction between bits in a given
209 string. The *in silico* generated data discussed in this commentary are composed of 10-bit
210 strings, each corresponding to the presence/absence of a different microbial taxa. Such
211 a case would have $2^{10} = 1024$ total combinations of taxa, and corresponding phenotypic
212 measurements (parasite load).

213

214 Similar to the 4-bit string example used to explain the method, note that each order has
215 a different number of possible combinations. That is, the number of insects that can
216 carry a combination of interacting taxa of a certain order. These are as follows: 0th = 1; 1st
217 = 10, 2nd = 45; 3rd = 120; 4th = 210; 5th = 252; 6th = 210; 7th = 120; 8th = 45; 9th = 10; 10th = 1. The
218 methods offered here measure every one of these interactions (e.g. all 210 of the possible
219 6th order interactions) between taxa. While our use of a 10-bit string (as opposed to an 8
220 or 15 bit string) is rather arbitrary, it is meant to highlight the vastness of the higher-
221 interaction problem: Even if we suspect that only 10 taxa are meaningfully influencing a
222 phenotype of interest (many studies contain more), the possible ways that these species
223 are interacting, and the number of measurable coefficients between them can be
224 astronomical in number.

225

226 Having outlined the method used to quantify higher-order interactions above, it is
227 important to directly explain the presumptive biological interpretation of the values.
228 The WHT returns a Walsh coefficient for each “order” of interaction. This corresponds
229 to the relative strength or importance of that “order” in the phenotype being measured.
230 Therefore, the Walsh-Hadamard Transform can help to interpret the overall presence
231 and eminence of higher-order interactions between taxa in a microbiota.

232

233 **Results**

234

235 Figure 2 depicts an *in silico* generated collection of 1024 insects, each containing one of
236 the combinations of 10 taxa ($2^{10} = 1024$), organized into a fitness graph (see

237 Supplemental Information for details on the in silico code and dataset). Each individual
238 also has a parasite load. While other statistical methods may not require all possible
239 combinations of taxa in order to extract meaningful information on the magnitude of
240 higher-order interactions, creating the combinatorial set demonstrates the size and
241 shape of the problem, all of the possible ways that taxa could interact.

242

243 Figure 3 depicts the raw calculations of the Walsh coefficients for all of the higher-order
244 interactions (orders 2 – 9). Here we observe that the magnitude and direction of the
245 interaction effect (Walsh coefficient) varies across different combinations of taxa. That
246 the Walsh Hadamard Transform can disentangle these types of effects is a feature of the
247 calculation and reveals the possibilities that exist in complex systems—like the
248 microbiota—where many different objects are interacting. It is especially important to
249 note that the specific identity of the taxa present is very important to understand in
250 determining their interaction. We cannot assume that, for example, all third-order
251 interactions (interactions between three taxa) will have the same magnitude or direction
252 of interaction (e.g. positive or negative).

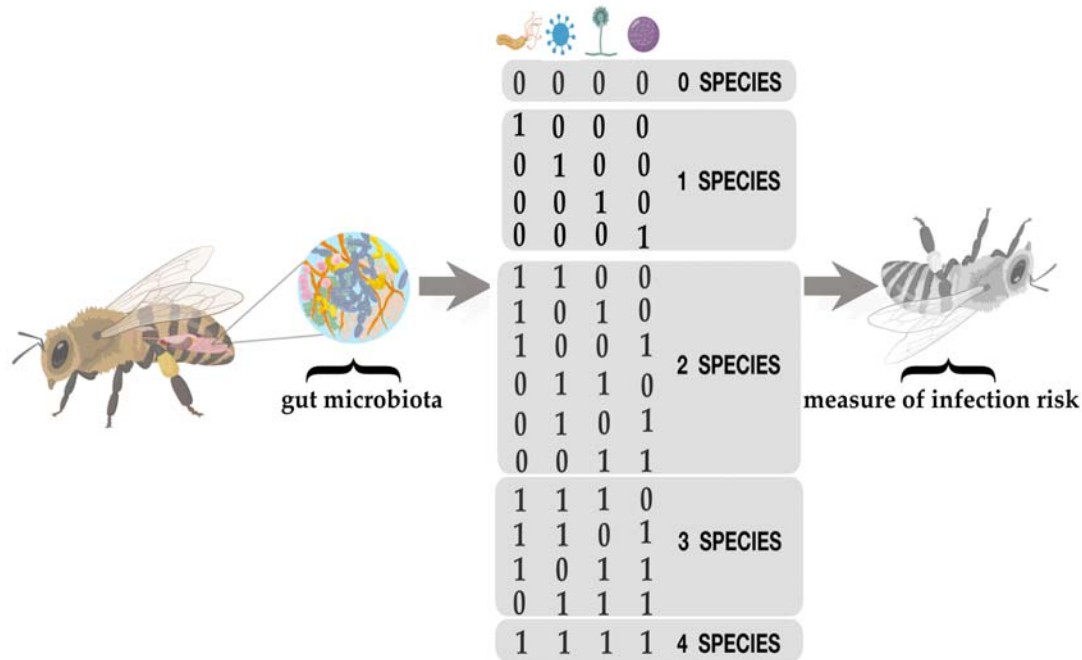
253

254 Figure 4 demonstrates the sum of the absolute values of the interaction coefficients
255 highlighted in Figure 3. Here, we can observe the raw magnitude (leaving the sign—
256 positive or negative—of it aside) of higher-order interactions as a function of interaction
257 order. Between 1st and 9th order, higher-order effects increase, suggesting that they
258 become more *meaningful* with the number of interacting microbes. Without knowing

259 the specific mechanism at work, determining the mean magnitude of coefficients
260 provides relevant information on the eminence of a given order in the microbiota. For
261 example, in our *in silico* microbiota the 9th order taxa represents the highest magnitude
262 of interaction relative to other taxa orders (Figure 4). As this is a theoretical, *in silico*
263 generated microbiota, we can interpret this finding as meaning that 9th order
264 interactions contain the largest average deviation from additivity. That is, knowledge of
265 how any given 9 taxa will interact requires very specific information on the identity of
266 which 9 taxa are interacting. This is a characteristic of a highly non-linear, complex
267 systems.

268

269 Note that all of these values—the raw *in silico* parasite load data, the interaction
270 coefficients for all individual interactions, and the scaled, absolute value coefficients—
271 can be found in the Supplementary Material.

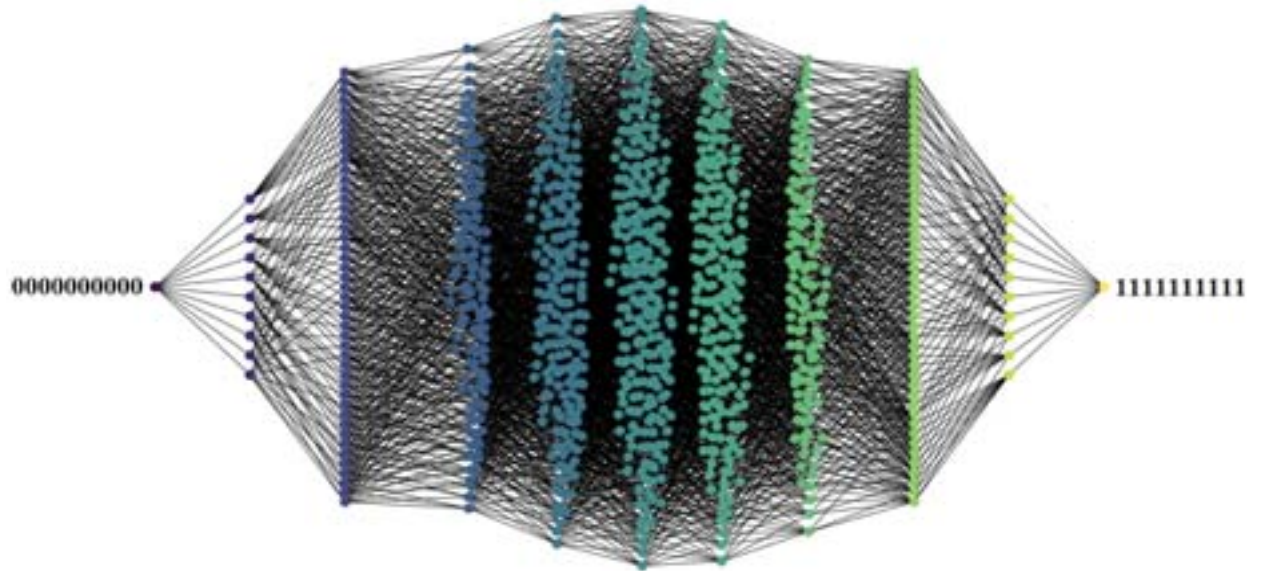


272

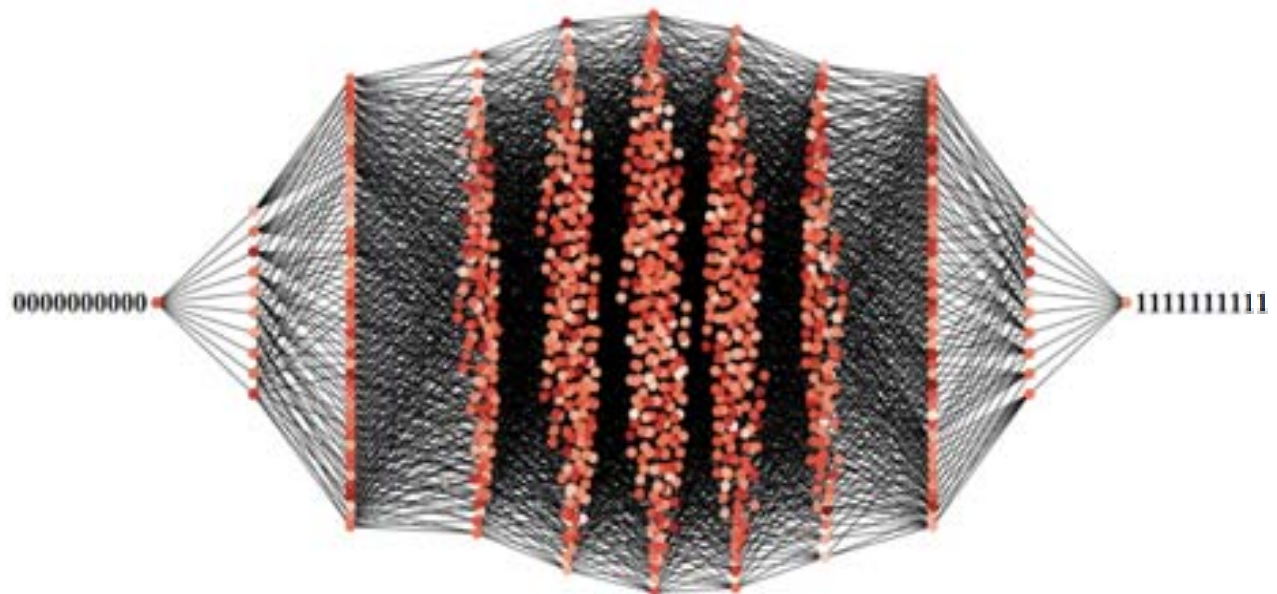
273 Figure 1. Schematic representation of higher-order interactions in the insect gut
 274 microbiota. We represent the presence of microbial species in the gut similarly to the
 275 presence of a genetic locus. Species composition are represented in binary strings. In
 276 this configuration, the combination 0011 represents both the presence and absence of
 277 two species. For each string combination, we associated a phenotypic measurement,
 278 such as infection risk. We quantify “epistatic” interactions between microbes in n
 279 dimensional space, where n represents the number of species interacting.

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281



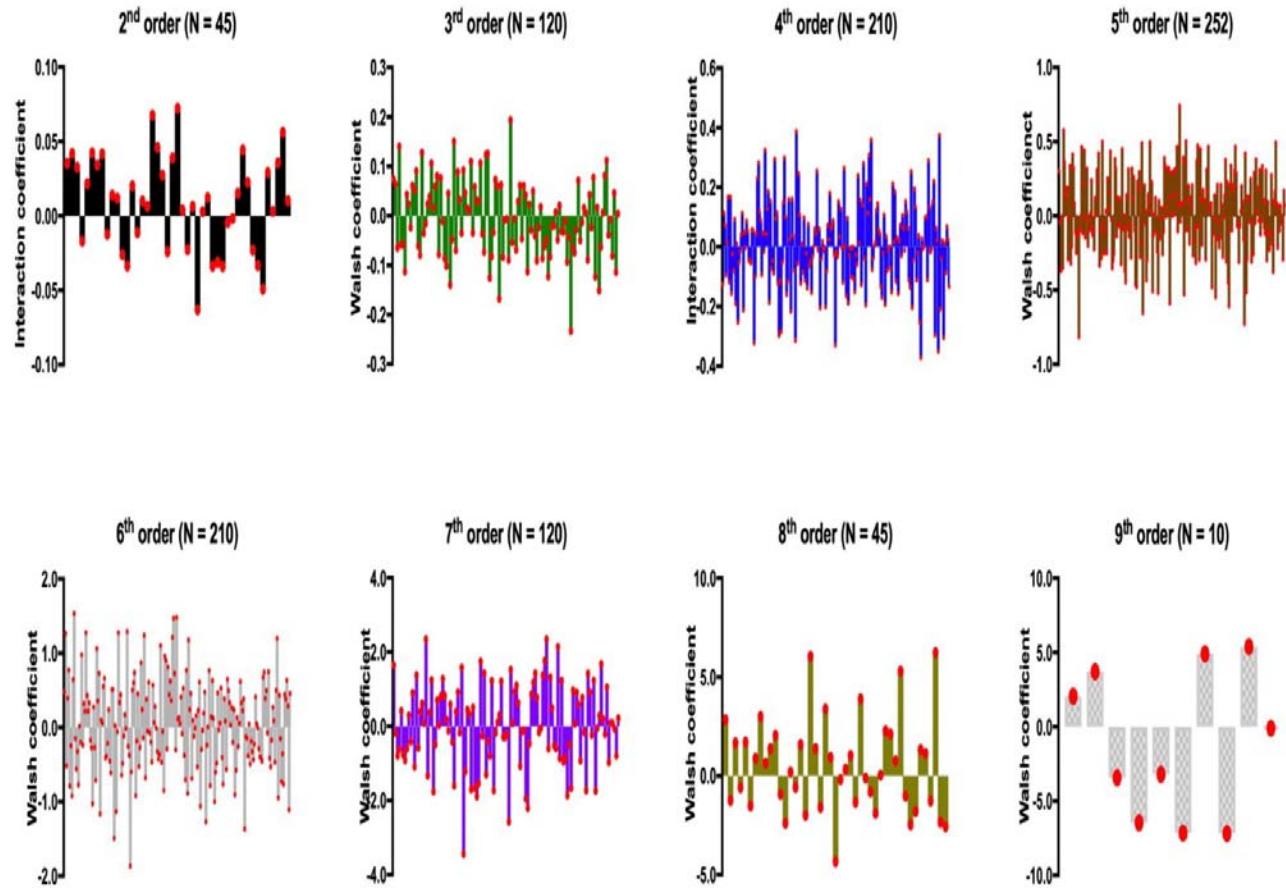
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284 Figure 2. An *in silico* generated microbiota with a combinatorial complete set of 10 taxa,
285 composing 1024 total nodes (2^{10}). Edges link neighboring strings. In genetics, these edges
286 represent genotypes that are mutational neighbors; in this data set they just denote
287 insect microbiomes that differ by the presence/absence of a single taxa). (A) Color
288 gradient represents the mutational class (order): lighter colors correspond to higher
289 orders (5 taxa, 6 taxa, etc). (B) Color gradient represents actual parasite load
290 measurements. Darker colors represent higher parasite loads.

291

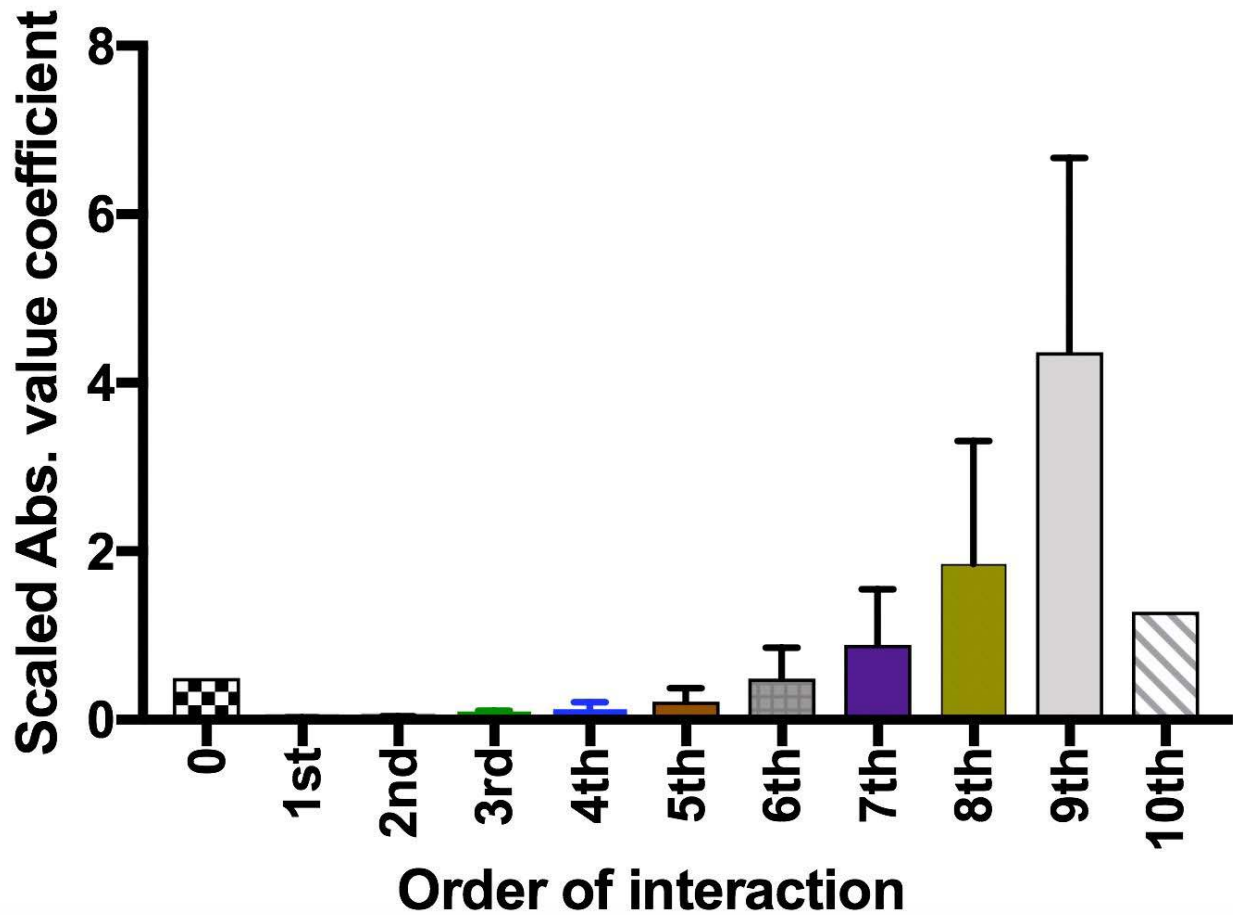


292

293 *Figure 3. The entire space of higher-order interactions between taxa in all possible combinations,*
294 *organized by higher-order class. Each line represents an individual combination of the 10*
295 *taxa, and the y-axis is the epistatic coefficient corresponding to that taxa. That is, for*
296 *1001000001, there is a value corresponding to the magnitude of the 3rd order epistatic*
297 *interaction between the presence of the taxa corresponding to first site, fourth site, and*
298 *tenth site. 0th and 10th orders are missing from this figure because they are composed of a*
299 *single interaction. 1st order is missing because it doesn't constitute "higher-order"*
300 *interactions, but rather, the "main effects" of each taxon acting in isolation.*
301 *Alternatively, higher order interactions (2nd through 9th) are all composed of multiple*
302 *parcels interacting. Please note that the y-axes are different across figures.*

303

304



305

306 Figure 4. *The magnitude of interaction between taxa.* Absolute value, averaged magnitude
307 interactions across interaction orders. These are the averaged, scaled, absolute values of
308 the data show in Figure 3. The purpose of this depiction is to illustrate how the
309 magnitude (not sign) of the interactions change with interaction order. In this *in silico*
310 microbiota data set, this translates to the 9th order interactions (interactions between 9-
311 different taxa) containing the most non-linearity, overall.

312

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319 **Discussion**

320 In this commentary, we explore the possibility of higher-order interactions between
321 taxa composing an insect gut microbiota. Using *in silico* and applied mathematical
322 approaches, we demonstrate how higher-order interactions can be measured in a
323 complex system of interacting microbial taxa. In our theoretical scenario, higher-order
324 interactions are present and generally increase in relevance with the order of
325 interaction. Though our results are theoretical, they are results nonetheless (Goldstein,
326 2018), highlighting the vast scope of the higher-order interaction problem, and outline
327 one method that can be used to deconstruct them in biological systems. Though
328 empirical data of the size and scope used in this study are currently challenging to
329 generate, this intractability may be temporary, and future methods may permit the
330 generation of data similar in structure to those explored in our theoretical examination.

331

332 The approach used in this study—the Walsh-Hadamard Transform—has been
333 previously used by theoretical population geneticists to measure non-linear interactions
334 between mutations (Weinreich et al., 2013). Several empirical data sets in genetics and
335 genomics have demonstrated that the sign of interaction effects can change readily with
336 the identity of the interacting parcels (Guerrero et al., 2019; Weinreich et al., 2018a, 2013).
337 Given this, we predict that the taxa that compose the gut microbiota might be similarly
338 defined by higher-order interactions. The capacity for measuring the effects of higher-
339 order interactions on host fitness is an important step towards understanding the effects

340 of microbiota on their host. Indeed, considering higher-order interactions can enable
341 more robust information on non-linear interactions in microbiome communities.

342

343 We found that higher-order interactions were present, and that taxa interacted both
344 positively and negatively. Combined interactions among taxa are augmented compared
345 to what is expected from individual effects when phenotypic effects are positive. In
346 contrast, higher-order effects are negative when combined interactions among taxa
347 show a diminished return and are less fit than would be expected from their individual
348 effects (fig 3). Such combinatorial complete data-sets can tell us what scale microbial
349 interactions matter in predicting host infection. Moreover, they reveal patterns of
350 interactions, particularly those combinations that interact synergistically or
351 antagonistically (Hartl, 2014). One potential limitation of the outlined approach is the
352 requirement for combinatorial complete datasets. For high-diversity microbiomes,
353 including humans and plants, it is not currently feasible to carry out experiments
354 measuring phenotypes for all the possible microbial interactions.

355

356 Microbe-mediated protection against pathogens depends on subtle differences in gut
357 community structure. In North American wild bumble bees, lower *Chrithidia* parasite
358 infection loads are associated with higher microbiota diversity. Using transplants to
359 naive host, it was shown that the core-gut bacteria were responsible for conferring
360 resistance to the *Chrithidia* parasite, while non-core gut bacteria were found to be less
361 effective against the parasite (Mockler, Kwong, Moran, & Koch, 2018). In mosquitos, gut

362 bacterial species can trigger an immune defense against *Plasmodium* parasites, the
363 causative agent of malaria (Bahia et al., 2014). In sandflies, highly diverse midgut
364 microbiota's were found to be negatively correlated with the parasite that causes the
365 vector-borne disease leishmaniasis (Kelly et al., 2017). While these studies did not
366 investigate the effects of higher-order interactions on host fitness, future experimental
367 studies manipulating microbial communities should consider combinatorial designs.

368

369 Recent theoretical work suggests that higher-order modeling approaches are able to
370 capture volumes of rich data arising from complex ecological interactions (Lambiotte,
371 Rosvall, & Scholtes, 2019b). In this perspective, we have adapted approaches from
372 population genetics to the study of host-associated microbiota. Applying these methods
373 to the analysis of real experiments will yield important insight into microbiome
374 dynamics, towards a richer understanding of just how peculiar the microbiota is, and
375 the many meaningful interactions that it embodies.

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382 and Walsh-Hadamard primer. We finally thank Lawrence Uricchio for constructive
383 feedback on our manuscript.

384

385 **Data Availability**

386 The *in silico* data used in this study and code used to generate them can be found on

387 github: <https://github.com/OgPlexus/MicrobeTaxa1>

388

389 **Supplemental Information**

390 The authors have prepared a simple mathematical primer on the Walsh-Hadamard

391 Transform: <https://github.com/OgPlexus/MicrobeTaxa1>. For a more rigorous

392 understanding, readers are encouraged to engage the works cited in this manuscript.

393

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395

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