Drosophila Undigested Metabolite Profiling reveals age related loss of intestinal amino acid transport regulates longevity

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Abstract

Age-related intestinal decline is marked by altered epithelial architecture, loss of barrier function, elevated stress and immune signalling and changes to the intestinal microbiota. Despite this we do not yet know whether age-related intestinal decline impacts nutrient management, a key function of the intestinal epithelium.

In this study we have developed *Drosophila* Undigested Metabolite Profiling (D.U.M.P.) to assess the impact of intestinal ageing on nutrient absorption/excretion balance. We demonstrate that ageing results in a significant increase in amino acid load in the faecal matter that is largely driven by the microbiota and shortens lifespan. Increased amino acid load is associated with reduced expression of a subset of amino acid transporters. Knockdown of the amino acid transporter *slimfast* in the intestinal epithelium extends lifespan and confers improved microbial control in aged flies, suggesting reduced transporter expression is protective, preventing cellular uptake of excess amino acids.

We conclude that age-related changes to the microbiota are an important determinant of the local nutritional environment, with consequences for health. In addition, age-related decline of the intestinal epithelium may impact its capacity for nutrient absorption. These findings have significant implications for the rational design of anti-ageing nutritional therapies.

Introduction

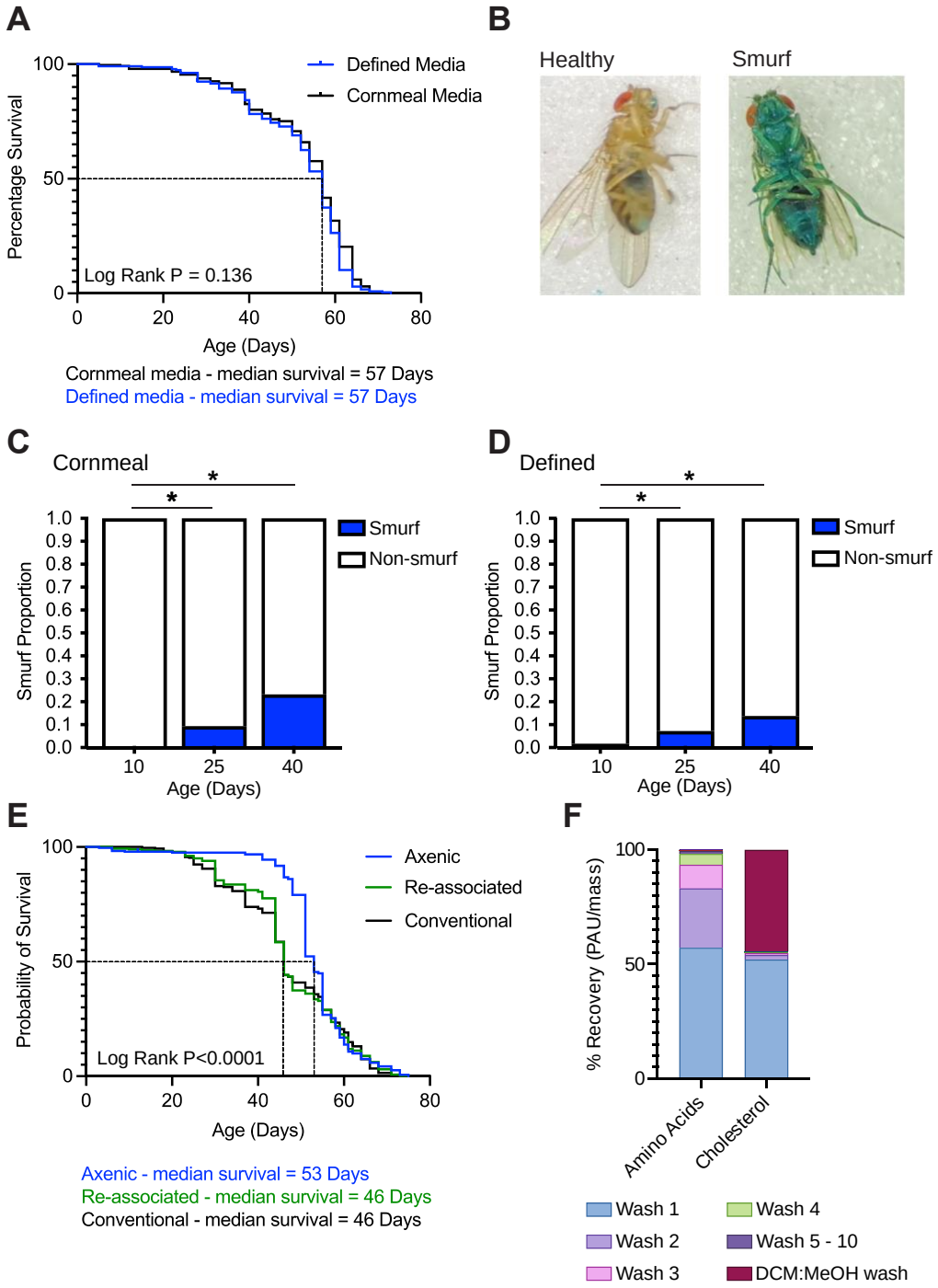
The ageing intestine has been the subject of intensive study in recent years with significant advances in our understanding of the molecular mechanisms that underpin intestinal homeostasis. Ageing results in a loss of intestinal epithelial homeostasis characterised by changes in architecture, cellular composition, and regenerative potential of the stem cells[1–5]. In addition, immune activation and intestinal permeability increase and there are shifts in the size and taxonomic composition of the commensal microbiota[6–15]. These phenotypes are interrelated such that interventions that target one will impact other aspects of intestinal homeostasis. Moreover, preventing a loss of intestinal homeostasis with age delays age-related decline in the organism as a whole[3,6–8,14,16–21]. Collectively, these studies have established the importance of intestinal homeostasis as a cornerstone of healthy ageing.

Alongside these studies of intestinal cell biology, physiology and microbiota we have seen growing evidence of the role of nutrition in maintaining health throughout the life course. The signalling network that regulates metabolism and growth is well accepted to influence lifelong health. This network responds to nutritional cues, and dietary nutrients and feeding patterns can themselves regulate ageing (reviewed in Longo and Anderson, 2022[22]). It is clear from this body of work that interactions between nutritional cues and longevity-associated signalling pathways are complex and context dependent. Genetics, sex, existing health status and age of onset all influence the ability of dietary interventions to extend lifespan and improve health (reviewed in Longo and Anderson, 2022[22]). Despite the challenge posed by this complexity, personalised nutrition has the potential to optimise individual health and longevity.

One key outcome of the intensive study of nutritional regulation of ageing is an appreciation that nutritional needs change significantly with age[23] as does the ability of established nutritional interventions to extend longevity[22]. Taking this together with our knowledge of age-related intestinal decline it is surprising that few studies have addressed the impact of intestinal decline on nutrient management, a key function of the intestinal epithelium. In this regard, older animal studies suggest a reduction in transport of a range of nutrients with age[24–26]. More recently, it has been postulated that age-related defects in membrane trafficking drive loss of intestinal barrier function and might also disrupt nutrient

65 absorption, thus underlying age-related changes in metabolic status[27]. An assumption that
66 nutrient absorption is reduced with age pervades online sources of nutritional advice, and
67 while these claims are not unfounded it has not yet been established that a loss of nutrient
68 absorption is a general feature of ageing or whether such a loss impacts all nutrient types.
69 More importantly, the mechanisms underlying such a loss, the consequences for intestinal
70 physiology and ageing, and the impact on the effectiveness of nutritional interventions have
71 not been explored.
72 A recent article summarizing the discussions that took place at an NIH workshop on
73 diet and healthspan put forward a list of questions that will need to be considered priorities
74 in future research, among them the question: "Do nutrient transport and uptake change
75 with age?"[28]. However, exploring this is challenging because to date, tools enabling deeper
76 investigation of age-related changes in intestinal nutrient management have been limited.
77 We aim to address this in *Drosophila melanogaster*, a short-lived and genetically tractable
78 model system that has been extensively used in studies of the ageing intestine. We present
79 a novel assay that combines a chemically defined diet with metabolic profiling of faecal
80 nutrient content to assess changes in nutrient uptake/excretion balance with age. Using this
81 assay, we demonstrate that amino acid load is increased in the faecal matter of aged flies,
82 with excess amino acids largely contributed by the microbiota. Further, age-related increase
83 in amino acid load is associated with reduced expression of a subset of amino acid
84 transporters in the midgut epithelium. Intestinal knockdown of the amino acid transporter
85 *slimfast* appears protective, extending lifespan and improving microbial control in aged flies.
86 These data highlight the contribution of age-related intestinal decline to the local
87 nutritional environment, with consequences for health. In addition, age-related decline may
88 drive changes in nutrient absorption capacity. Therefore, our findings have significant
89 implications for the rational design of anti-ageing nutritional interventions.
90
91 **Results**
92
93 **Drosophila Undigested Metabolite Profiling (D.U.M.P)**
94 To measure changes in nutrient absorption/excretion balance we adopted a strategy of
95 comparing nutritional input and output by combining a chemically defined *Drosophila*
96 media[29,30] with mass spectrometry profiling of faecal nutrient content. We included a non-

absorbable food dye in the media to calculate the quantity of food ingested for each faecal sample by measuring its dye content. This enabled direct comparison between nutrient intake and output.



Supplemental figure 1. Defined media does not impact lifespan. (A) Lifespan of Canton-S female flies fed during adulthood on cornmeal or defined media. N > 200 per condition at day 1 (B) Images displaying the characteristic smurf phenotype. (C-D) Proportion of smurf

105 flies within the total population taken at the ages shown for (C) cornmeal and (D) defined
106 media. N > 200 per condition at day 1 (E) Lifespan of axenic, re-associated and
107 conventionally reared flies fed during adulthood on defined media. N > 450 Canton-S
108 females per condition at day 1. Asterisks denote the result of binomial tests, where *
109 p<0.05. (F) Percent recovery; the sum of percent abundance (PAU)/mass of all amino acids
110 or cholesterol per wash, as a percentage of the sum of PAU/mass of all amino acids or
111 cholesterol for all washes. No cholesterol was detected following wash 5.
112
113 First, we needed to establish that the defined diet supported the adult stage of our
114 wild-type Canton-S flies as expected under our laboratory conditions. Given the previously
115 reported developmental delay of flies reared on the defined diet[29], we reared flies on our
116 standard cornmeal diet, transferring them to the defined diet in early adulthood. Diet is an
117 important determinant of lifespan and age-related health and so we assessed the lifespan of
118 our flies on the defined diet compared to our standard cornmeal diet. We found the change
119 in diet did not alter the lifespan (Fig. S1A). We also found that flies on the defined diet
120 continued to show an age-related increase in intestinal permeability (Fig. S1B-D) as
121 measured by the Smurf assay[10,31]. Finally, we assessed the ability of the defined diet to
122 support lifespan of axenic flies. We found axenic conditions resulted in lifespan extension
123 (Fig. S1E), demonstrating that the nutritional requirements of adult female flies are fully met
124 by the diet alone and do not need to be supplemented by the microbiota. In fact, the
125 presence of the microbiota is detrimental with regards to lifespan in Canton-S females
126 under our laboratory conditions.
127 Having confirmed the defined diet did not impact lifespan we developed a faecal
128 collection protocol (Fig. 1A). Inclusion of FD&C blue no. 1 in the media enabled us to score
129 "Smurf" flies that had lost intestinal barrier function and exclude them from the assay as the
130 concentration of dye in the fecal matter is no longer representative of food intake [10,31]. In
131 the remaining flies the concentration of blue no. 1 in each faecal sample then provides a
132 proxy for food consumed. Normalisation of mass spectroscopy data to dye concentration
133 controls for differences in food intake or the quantity of faecal matter collected.
134 For the purposes of this study, we quantified amino acids only from our final samples
135 (see below); however, we provide details of sample preparation designed to enable

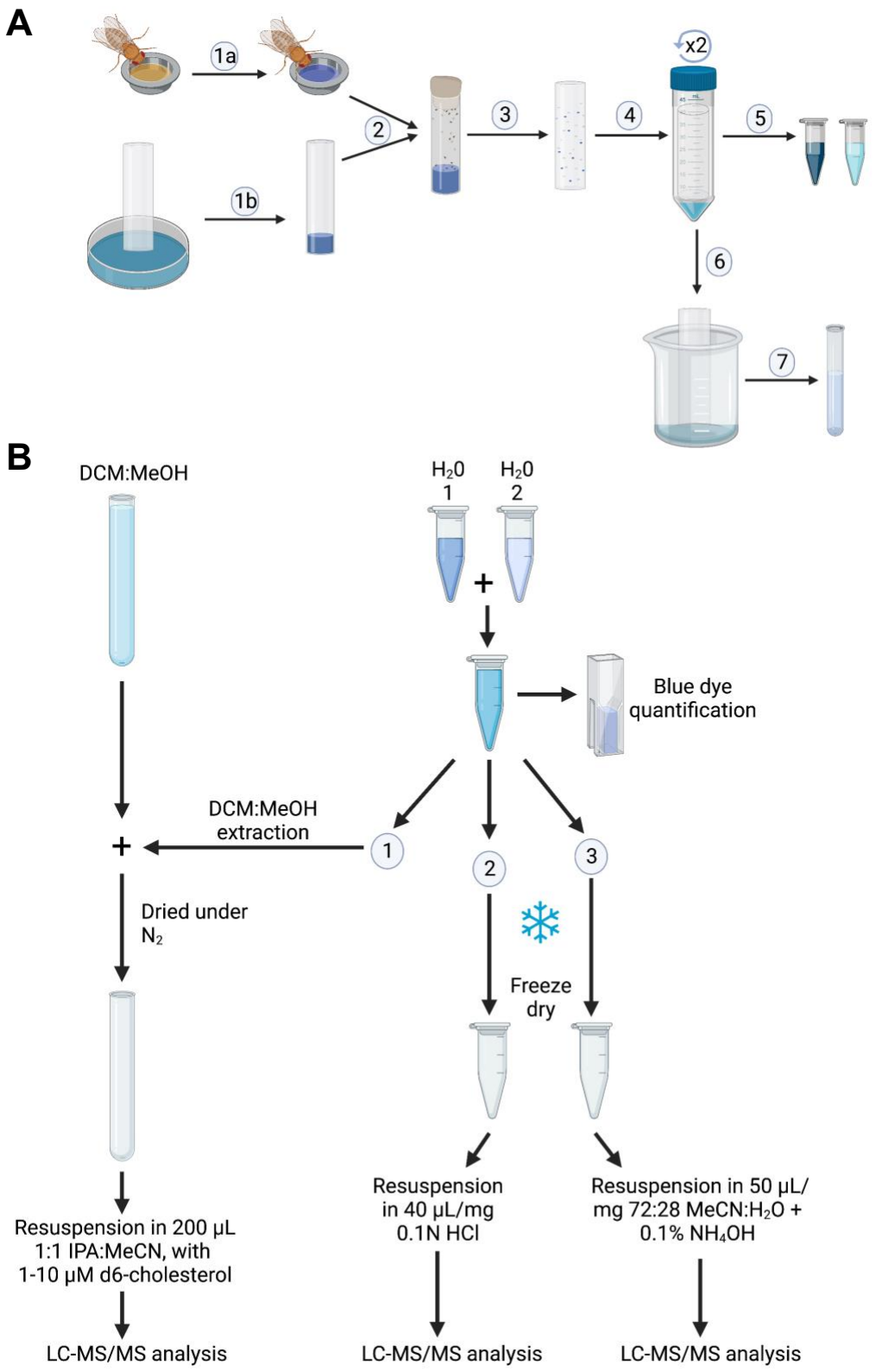


Figure 1. Schematic of Drosophila Undigested Metabolite Profiling assay. (A) Sample collection for the DUMP assay; 1a| Flies placed on blue food for 24h. 1b| Collection vials prepared. 2| 60 non-smurf flies transferred to collection tubes. 3| Flies incubated at 25°C for up to 5 hours, then anaesthetised and removed with food. 4| All collection vials washed

141 within falcon tubes with the same 1.5mL H_2O to produce a concentrated sample. Wash step
142 is repeated to give a second sample 5| water samples transferred to microfuge tubes and
143 stored at -80°C. 6| collection vials washed in 5mL 1:1 DCM:MeOH to collect non-polar
144 fraction. 7| DCM:MeOH sample collected in glass-storage tube. Sample stored upright at -
145 80°C. (B) Sample processing for the DUMP assay; the two water washes are combined and
146 1-2 µL removed for quantification of blue dye. The remainder is split three ways for 1.
147 Cholesterol, 2. Amino acid, and 3. Sugar analysis. DCM is added to the water fraction to
148 extract cholesterol. The DCM layer is added to the DCM:MeOH wash sample and dried
149 under a stream of N_2 before resuspension 1:1 IPA:MeCN. The amino acid and sugar samples
150 were lyophilised, then resuspended in 0.1N HCl or 72:28 MeCN:H_2O + 0.1% NH_4OH
151 respectively, prior to LC-MS/MS analysis. Full details of each step are given in the materials
152 and methods section. Created in Biorender.
153
154 measurement of amino acids, sugars and cholesterol from each faecal collection (Fig. 1B).
155 Faecal collection involved washing faecal matter from the sides of the collection vials. Initial
156 tests showed that two water washes were sufficient to collect 80% of amino acids and
157 almost 50% of cholesterol (Supp. Fig. 1F). Given the time required for each wash and the
158 diminishing returns from each additional wash, we used two water washes going forward.
159 An additional wash with DCM:MeOH enabled collection of the remaining cholesterol (Supp.
160 Fig. 1F), and presumably aids in collection of non-polar molecules more generally. We also
161 found the DCM:MeOH wash to contain some amino acids, including cysteine which was not
162 detected in the water washes. Together our faecal collection and analysis protocols result in
163 samples that can be used to measure a wide range of nutrients and metabolites. This
164 protocol can also be adapted for untargeted metabolomics approaches.
165
166 **Elevated amino acid levels in the faeces of aged flies originate from the microbiota**
167 Given the importance of dietary protein levels in lifespan determination[32] and previous
168 reports of reduced amino acid transport with age[24], we next applied the D.U.M.P. assay to
169 assess changes in amino acid absorption/excretion balance with age. We found a consistent
170 trend toward increased amino acid concentrations in faecal matter from aged flies, although
171 not every amino acid showed a statistically significant change (Fig. 2A).

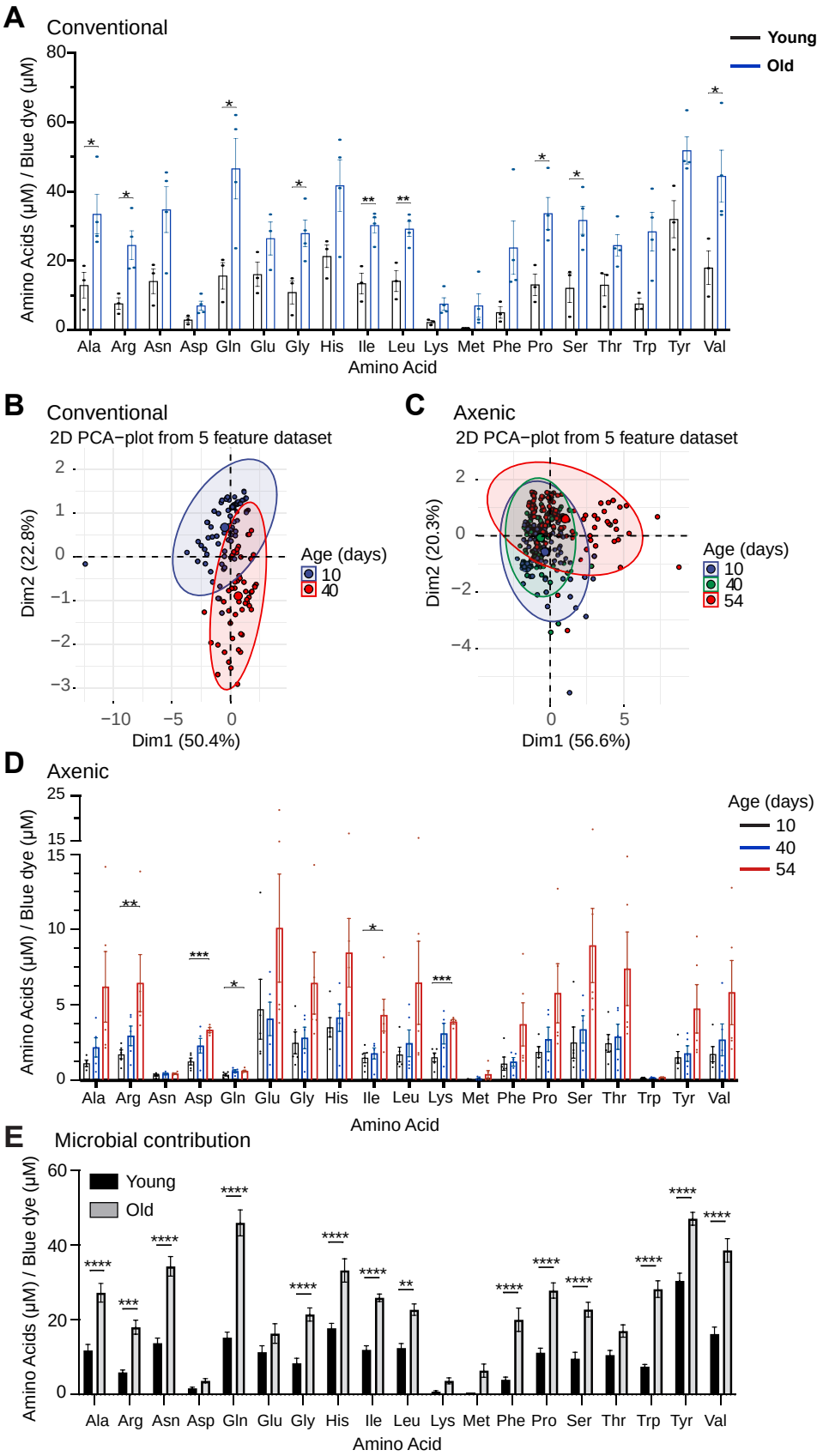


Figure 2: Drosophila Undigested Metabolite Profiling uncovers changes in nutrient egestion with age. (A) Relative concentration of amino acids in the faecal deposits of 10-day

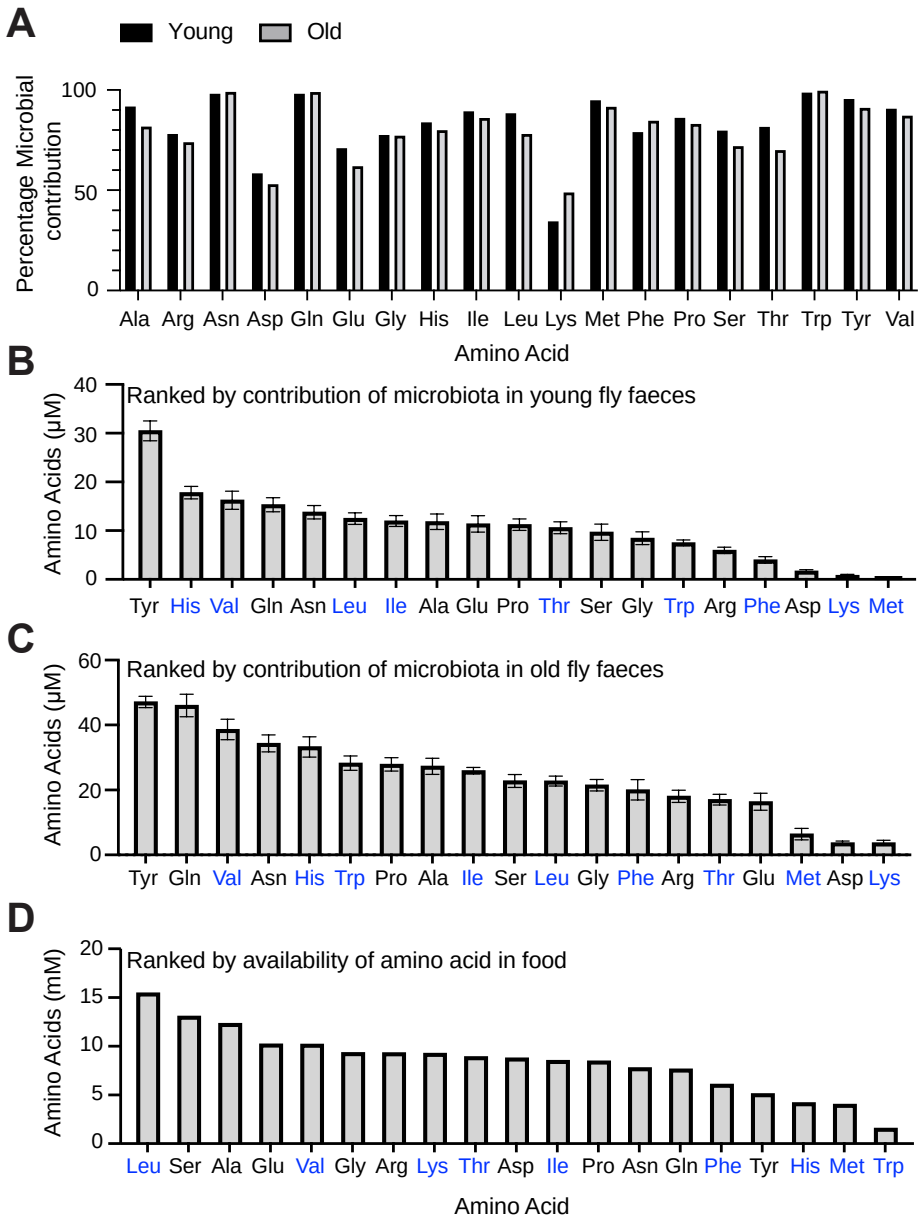
175 (9-11 day) and 40-day (39-44 day) flies, normalised to the concentration of blue food dye.
176 (B-C) Principal component analysis for the overall structure of amino acid data from (B)
177 conventional and (C) axenic samples plotted with respect to the first and second principal
178 components. (D) Relative concentration in the faecal deposits of 10-day (9-11 day), 40-day
179 (38-40 day), and 54-day (52-54 day) axenic flies, normalised to the concentration of blue
180 food dye. Each sample pooled from >300 mated female Canton S flies. N = 3 biological
181 replicate samples for the conventional dataset and N = 5 for the axenic data. E)
182 Concentration of faecal amino acids, normalised to the concentration of blue food dye,
183 contributed by the microbiota in young and old flies. The microbial contribution was
184 calculated for each amino acid at the young timepoint by; Conventional concentration (9-11
185 days) – axenic concentration (9-11 days), and at the old timepoint by; Conventional
186 concentration (38-40 days) – axenic concentration (52-54 days), using the concentration of
187 amino acid normalised to blue dye for each sample. For each amino acid, at each time point,
188 the calculation was carried out between every conventional and every axenic measurement.
189 Bar graphs show mean ± SEM. Asterisks denote the results of 2-tailed unpaired T-test
190 (normally- distributed data) or Mann-Whitney test (not-normally distributed data; (A) Trp
191 and Try, (D) Arg, Glu, Met, Phe, Ser and Val), where * P<0.05, **P<0.01, ***P<0.001. For the
192 axenic data tests compared 10- and 54-day samples.
193
194 We reasoned the previously reported age-related increases in microbial load in the
195 fly intestine[6,7,10,14] may drive this age-related increase in faecal amino acid concentrations.
196 To test the microbial contribution to age related changes, we repeated the D.U.M.P. assay in
197 a cohort of axenic flies. Given the lifespan extension seen in axenic flies we included a later
198 life faecal collection. To characterise the structure of the data we performed a principal
199 component analysis (PCA) on the conventional and axenic datasets. Plotting the data in
200 respect to the first and second principal components, which explain the most variance,
201 showed clustering by age in the conventional dataset (Fig. 2B). In the axenic dataset there
202 was no separation between the 10 and 40-day faecal samples, indicating a high degree of
203 similarity between these samples. However, the sample collected at 52-days shows
204 separation from the 10 and 40-day samples (Fig. 2C). This is consistent with the fact that the
205 axenic lifespan shows no increase in mortality at the 40-day timepoint, while the
206 conventional lifespan is at 75% survival at 40 days (Supp. Fig. 1E). The axenic lifespan drops

207 rapidly from 80% to 50% survival between days 50 and 53 (Supp. Fig. 1E), suggesting the 52-
208 day timepoint is a closer match to the 40-day conventional sample with regards to biological
209 age. For this reason, the 52-day sample was used for further analysis.

210 To determine the contribution of epithelial ageing to the changing faecal amino acid
211 profile in the absence of the microbiota we compared our axenic samples across the age
212 range. As suggested by our PCA analysis (Fig. 2C), a trend toward increased amino acid
213 concentrations in the faecal matter of aged flies remained in the axenic cohort, although
214 this was statistically significant for fewer amino acids than in the conventional dataset (Fig.
215 2D). The amino acids that showed statistically significant changes upon ageing were
216 different between the conventional and axenic datasets and overall amino acid levels were
217 much lower, demonstrating the contribution of the microbiota. Nevertheless, age-related
218 changes in the intestinal epithelium are sufficient to drive increased amino acid load in aged
219 fly faeces.

220 In order to better understand the relative contributions of epithelial changes and the
221 microbiota to the overall aged amino acid load, we calculated the amino acid concentration
222 contributed by the microbiota in young (10-day conventional – 10-day axenic) and old (40-
223 day conventional – 52-day axenic) flies. This calculation was performed by comparing every
224 conventional value to every axenic value, for each amino acid. This analysis confirmed that
225 the microbiota is an important contributor to the faecal amino acid profile in both young
226 and old flies and that the microbiota contributes significantly more amino acids to the faecal
227 amino acid pool in old flies (Fig. 2E). Considering the microbial contribution as a percentage
228 of the total amino acid load in young and old flies demonstrated that, with the exception of
229 aspartate and lysine, the microbiota contributes over 70% of the total amino acid load
230 (Supp. Fig. 2A). Importantly, the percentage contribution changes little with age suggesting
231 that increased amino acid levels in aged flies are largely driven by age-related changes to
232 the microbial population and may reflect the increased microbial load typical in aged flies.
233 To visualise age-related changes in microbial contribution of specific amino acids we
234 ordered the amino acids by level of microbial contribution. Several changes in graph
235 position are seen between young (Supp. Fig. 2B) and old (Supp. Fig. 2C) flies, confirming that
236 changes in the microbial population contribute to age-related changes in the faecal amino
237 acid profile as well as to amino acid levels. In addition, the order of amino acids in both

young and old samples are different from that in the media (Supp. Fig. 2D), confirming the overall contribution of the microbiota to the faecal amino acid pool.

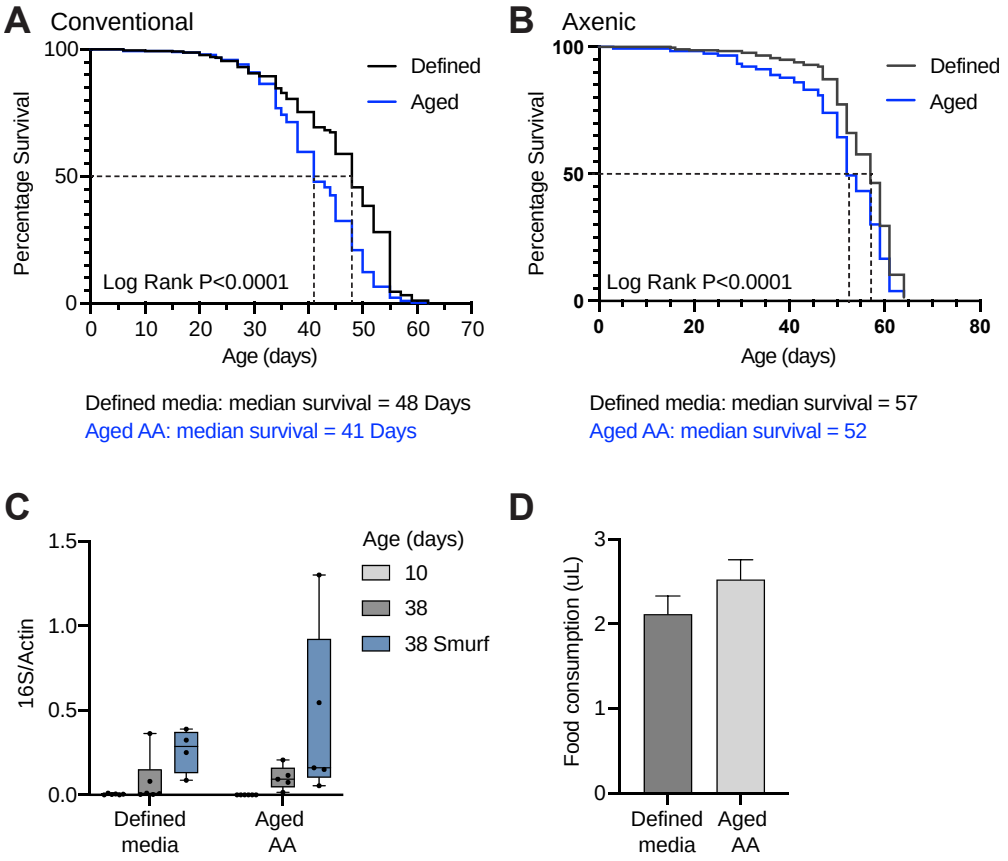


Supplemental figure 2: Microbial contribution with age shifts faecal amino acid profile. (A) The mean microbial contribution (Fig. 2E) shown as a percentage of the mean total amino acid load (Fig. 2A) for each amino acid in both young and old flies. (B-D) Amino acids ordered by level of microbial contribution in (B) young and (C) old flies, and by (D) availability within the food. Blue amino acid labels denote essential amino acids. Bar graph shows mean ± SEM. Asterisks denote the results of a 2-way Anova, where * P<0.05, **P<0.01, *** P<0.001, ****P<0.0001

249 **Recapitulating aged amino acid load shortens lifespan**

250 We assume the elevation in amino acid load in the faecal matter from aged flies reflects
251 elevated amino acid levels in the aged intestinal lumen. We next assessed whether these
252 changes altered lifespan. We used the D.U.M.P data to design a diet that mimics the amino
253 acid changes that occur with age in conventionally reared flies. The amino acid
254 concentrations in this diet, called Aged Amino Acid (AA) media, were determined by adding
255 the difference in mean concentration between the 10- and 40-day conventional faecal
256 samples to the concentration in the original defined media. Amino acid concentrations in
257 the Aged AA media ranged from 1.15 to 5.18 times the concentrations in the standard
258 defined diet. The average increase in concentration was 2.15 times that of the standard
259 diet, equivalent to dietary protein levels that have previously been shown to reduce
260 *Drosophila* lifespan[33].

261



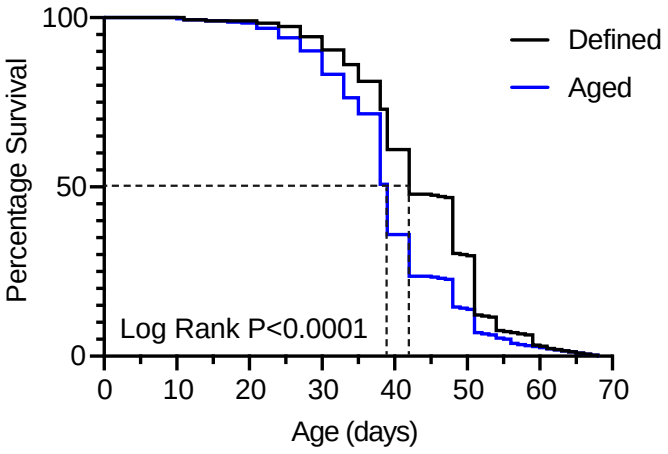
262

263 **Figure 3: Aged AA media reduced lifespan independent of microbial status.**

264 (A) Lifespan of conventionally colonised flies on defined media or Aged AA media. Dashed
265 black line denotes the median survival, n > 240 flies per condition at day 1. (B) Lifespan of
266 axenic flies on defined media or Aged AA media, n > 240 flies per condition at day 1. (C)

Internal bacterial load of young (10 day), old (38 day) and old smurf (38 days with intestinal barrier dysfunction) flies measured using universal primers for the 16s rRNA gene, normalised to actin. Data from at least 5 flies/sample, with a minimum of 6 samples. Box plots show the 25-75th percentiles and the median with the whiskers extending from min-max. (D) 48-hour food consumption (µL) of 9-10 groups of 10 flies fed liquid defined media or Aged AA media (omitting cholesterol). Bars show mean and standard error. No significant difference by unpaired t-test. All experiments were performed on mated Canton-S female flies.

We found that exposure to the Aged AA media from young adult life significantly and reproducibly reduced lifespan (Fig. 3A, supp. Fig. 3A). This result was also observed in a cohort of axenic flies, suggesting it is independent of the microbiota (Fig. 3B). Consistent with this observation we found no significant impact of diet on internal bacterial load (Fig. 3C). In addition, a capillary feeding assay[34], showed no significant difference in food intake between the standard and Aged AA media (Fig. 3D). These data are consistent with the known lifespan impact of a high protein diet[32] and suggest that increased intestinal amino acid load may contribute to age-related decline.



Defined media: median survival = 42 Days
Aged AA: median survival = 39 Days

Supplemental Figure 3: Aged AA media reduced lifespan

An independent replicate lifespan of conventionally colonised mated Canton-S females on defined media or Aged AA media. Dashed black line denotes the median survival, n > 240 flies per condition at day 1.

290 **Expression of intestinal amino acid transporters changes with age**

291 We next sought to understand why epithelial ageing results in increased faecal amino acid
292 levels in the absence of the microbiota. We reasoned that this may reflect reduced amino
293 acid transport across the intestinal epithelium with age, as previously reported for several
294 animal species[24]. As a proxy measure of transport capacity, we assessed the expression
295 levels of a panel of amino acid transporters in young and old intestinal tissue.

296 First, we generated a list of validated and predicted Drosophila amino acid
297 transporters that contained 48 genes, 21 of which were unnamed. We selected for gut
298 expression using online databases Flygut-*seq*[35] and FlyAtlas 2[36] and identified genes showing
299 homology to human intestinal amino acid transporters using DIOPT Ortholog Finder[37,38]. This
300 generated a final list of 18 genes spanning 8 solute carrier families (Supp. Table 1).

301

302 **Supplemental Table 1:** Final list of genes with validated or predicted amino acid transporter
303 function and predicted intestinal expression. Top human orthologs and weighted DIOPT
304 score determined from https://www.flyrnai.org/cgi-bin/DRSC_orthologs.pl. The genes in
305 grey font were not taken forward due to poor qPCR primers. *All top orthologs, sharing the
306 same weighted DIOPT score.

Drosophila Gene	Annotation Symbol	Homologous human Solute carrier family	Top Human ortholog	Weighted DIOPT score
Eaat1	CG3747	SLC1	SLC1A3	9.85
CD98hc	CG2791	SLC3	SLC3A2	5.82
CG1698	CG1698	SLC6	SLC6A16	3.94
Naat1	CG3252	SLC6	SLC6A16	3.94
CG13796	CG13796	SLC6	SLC6A14	3.94
Slimfast	CG11128	SLC7	SLC7A1	12.84
CG5535	CG5535	SLC7	SLC7A1	10.92
CG7255	CG7255	SLC7	SLC7A2	8.75
Minidiscs	CG3297	SLC7	SLC7A7	8.88
Sobremesa	CG9413	SLC7	SLC7A9	13.75
Kazachoc	CG5594	SLC12	SLC12A4	14.75
dmGlut	CG5305	SLC17	SLC17A3	5.89
CG43693	CG43693	SLC36	SLC36A1	9.9
Pathetic	CG3234	SLC36	SLC36A1-4*	5.92
CG8785	CG8785	SLC36	SLC36A4	7.87
CG1139	CG1139	SLC36	SLC36A1-4*	6.85
CG30394	CG30394	SLC38	SLC38A10	10.73

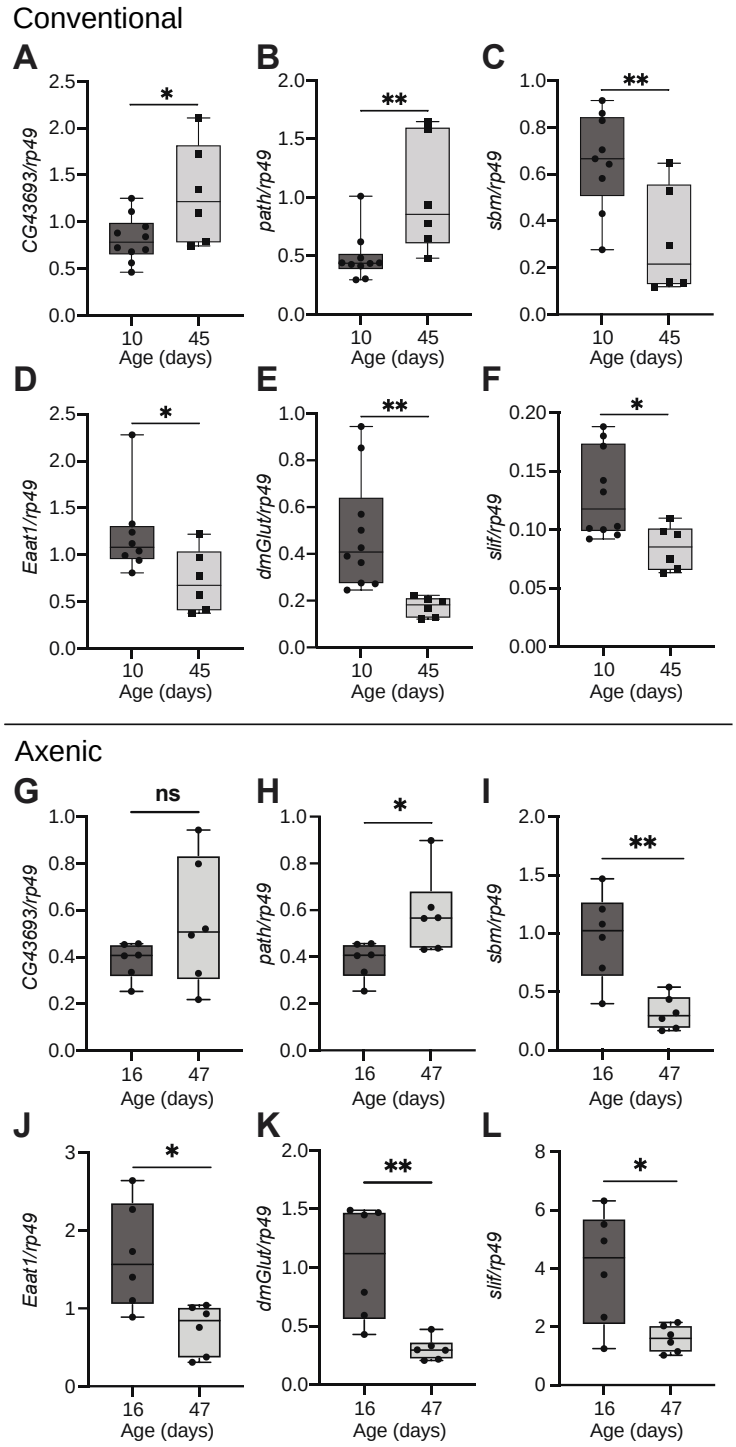
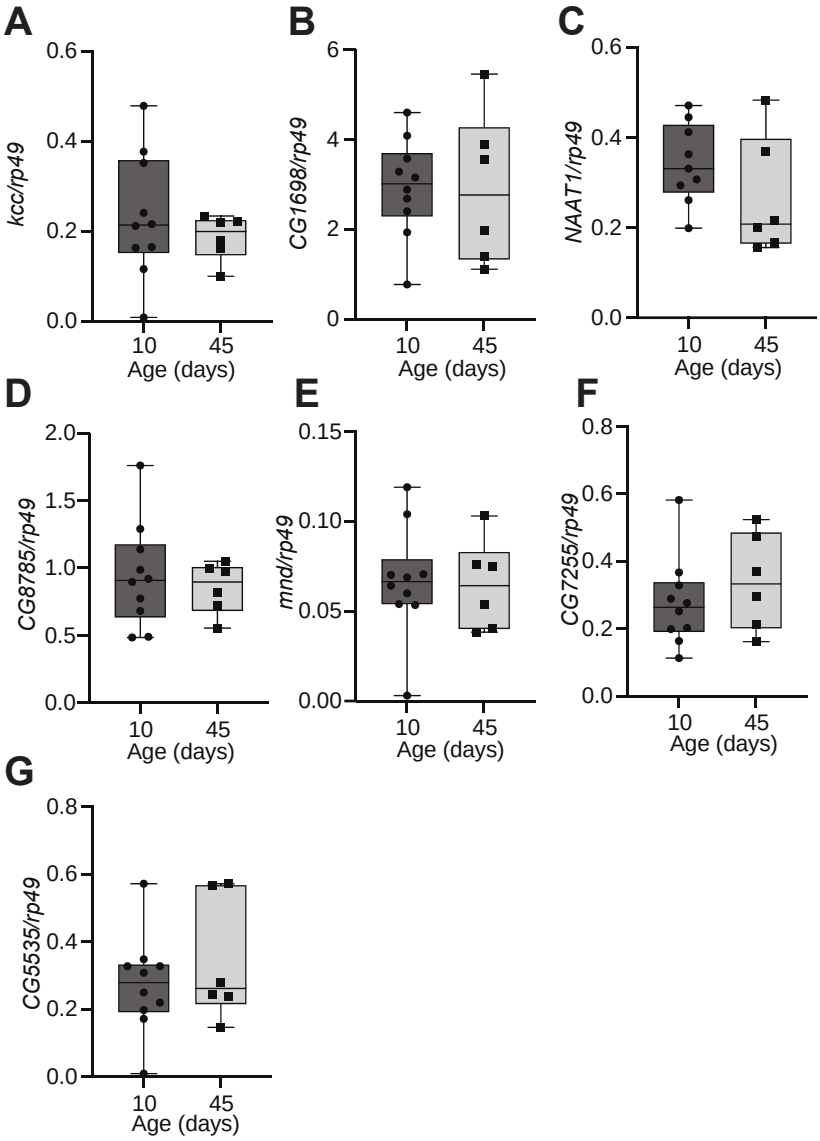


Figure 4: Age-related changes in amino acid transporter expression. (A-F) Transporter levels measured in dissected midguts from conventionally reared Canton-S females at 10 and 45 days of age. Normalised to the expression of rp49. N = 5 guts per sample, 8-10 samples at day 10, and 5-6 samples at day 45. (A) *CG43693*, (B) *Pathetic (path)*, (C) *sobremesa (sbm)*, (D) *Excitatory amino acid transporter 1 (Eaat1)*, (E) *dietary and metabolic glutamate transporter (dmGlut)*, (F) *Slimfast (Slif)*. (G-L) Transporter levels measured in dissected midguts from axenic Canton-S females at 16 and 47 days of age. Normalised to the

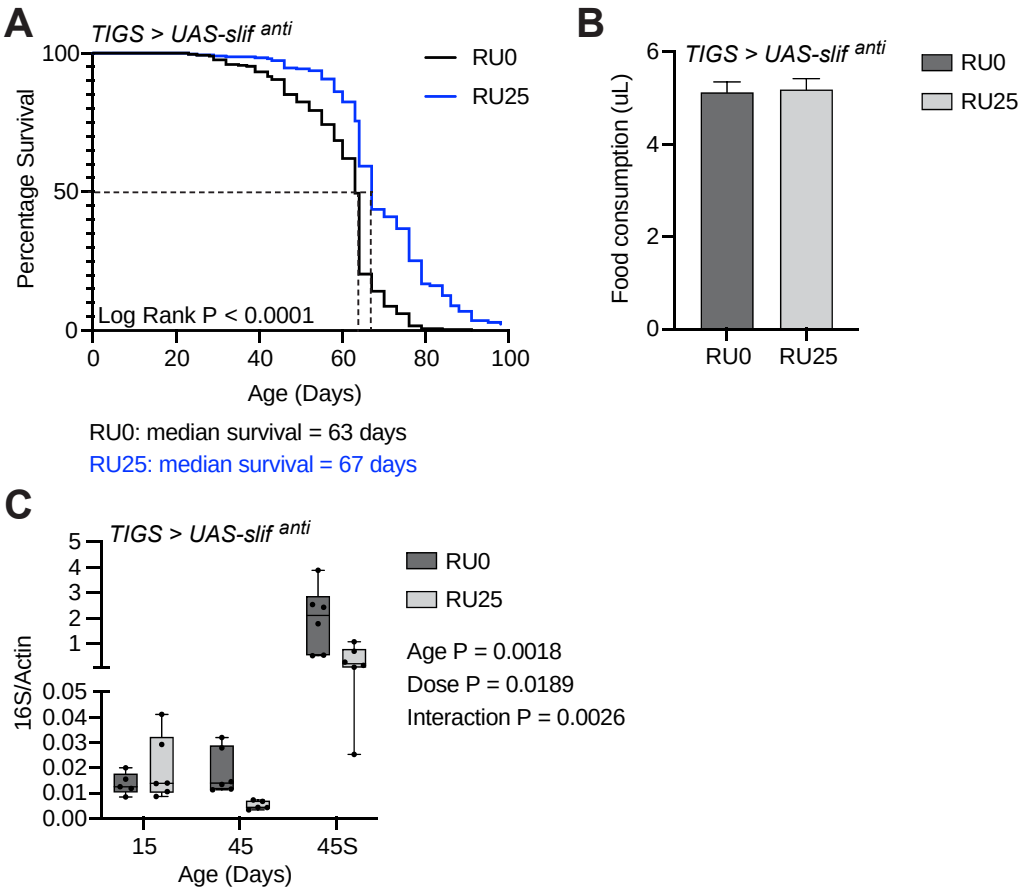
315 expression of rp49. N = 5 guts per sample, 5-6 samples at each timepoint. (G) *CG43693*, (H)
316 *Pathetic (path)*, (I) *sobremesa (sbm)*, (J) *Excitatory amino acid transporter 1 (Eaat1)*, (K)
317 *dietary and metabolic glutamate transporter (dmGlut)*, (L) *Slimfast (Slif)*. Box plots show the
318 25-75th percentiles and the median with the whiskers extending from min-max. Asterisks
319 denote the results of 2-tailed unpaired T-test (normally-distributed data) or Mann-Whitney
320 test (not-normally distributed data as calculated by the Shapiro-Wilk test; *path* and *Eaat1*
321 from conventionally reared flies (B,D)), where ns – not significant, * P<0.05, **P<0.01.
322
323 Of these we were able to validate quantitative PCR (qPCR) primers for 13
324 transporters, representing six solute carrier (SLC) families (Supp. Table 1). The validated
325 primers were were used to quantify expression in young (10 day) and old (45 day) Canton-S
326 female intestinal samples (Fig 4, supp. Fig 4). We found six transporter genes across 4 SLC
327 families that showed statistically significant changes with age: sbm, Eaat1, dmGlut and slif
328 showed significant downregulation, while CG43693 and path were upregulated (Fig 4). The
329 amino acid substrates for CG43693 (Fig. 4A) and Sombremesa (sbm) (Fig. 4C) are unknown,
330 and Pathetic (Path) (Fig. 4B) has been suggested to act primarily as a transceptor due to its
331 low transport capacity[39]. However, several amino acid transporters showed decreased
332 expression levels in aged intestines consistent with the D.U.M.P data.
333 Excitatory amino acid transporter 1 (Eaat1) (Fig. 4D) transports L-glutamate with
334 highest affinity, although L- and D- aspartate are also substrates, and there is evidence to
335 indicate a functional role in the intestine[40]. A second glutamate transporter, dietary and
336 metabolic glutamate transporter (dmGlut), also showed reduced expression with age (Fig.
337 4E). Both glutamate and aspartate show a trend toward increased levels in faecal samples
338 from old conventionally reared and axenic flies. This increase is statistically significant for
339 aspartate in axenic samples, suggesting the underlying cause is independent of the
340 microbiota.
341 Slimfast (slif) (Fig. 4F) transports L-arginine[41] and could be inferred to also transport
342 lysine due to its homology to the cationic amino acid transporter family, although this has
343 not been tested to our knowledge. A statistically significant increase in arginine and lysine
344 levels is seen in faecal samples from old axenic flies and for arginine in faecal samples from
345 old conventionally reared flies.

346 We further confirmed that the changes in expression reported for *path, sbm, Eaat1,*
347 *dmGlut* and *slif* were retained in axenic flies (Fig. 4H-K). CG43693 showed a trend toward
348 increased expression in old axenic flies, but this did not reach statistical significance. Overall,
349 this suggests the recorded changes in transporter expression are independent of the
350 microbiota. Taken together these data support a model whereby age-related changes in the
351 expression levels of amino acid transporters result in reduced transport across aged
352 intestinal epithelia which may contribute to increased luminal amino acid levels.
353



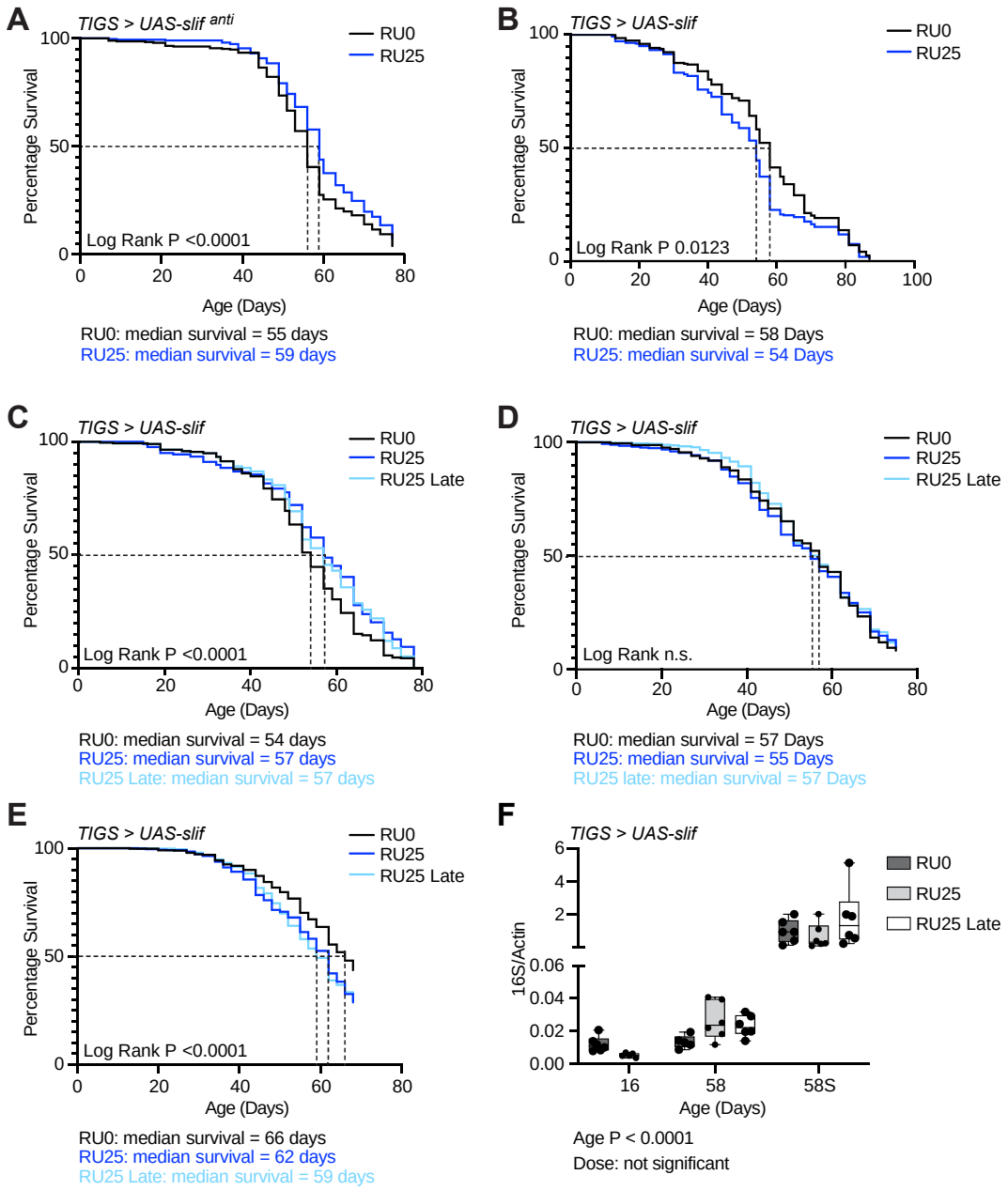
354
355 **Supplemental Figure 4: Some amino acid transporters do not show changes in expression**
356 **with age.** Transporter levels measured in dissected midguts from conventionally reared
357 Canton-S females at 10 and 45 days of age. Normalised to the expression of rp49. N = 5 guts
358 per sample, 8-10 samples at day 10, and 5-6 samples at day 45. (A) *kazachoch (kcc),* (B)

359 *CG1698*, (C) *Nutrient amino acid transporter 1 (Naat1),* (D) *CG8785,* (E) *minidiscs (mnd),* (F)
360 *CG7255,* and (G) *CG5535.* Box plots show the 25-75th percentiles and the median with the
361 whiskers extending from min-max.
362
363 **Intestinal slimfast expression level regulates lifespan**
364 We next sought to understand the physiological impact of reduced amino acid transporter
365 expression during ageing. We focused on slimfast due to the availability of genetic tools and
366 the significantly increased faecal concentration of its substrates with age.
367



368
369 **Figure 5: Intestinal *slimfast* levels regulate lifespan.**
370 (A) Lifespan of *TIGS > UAS-Slif^{anti}* females on defined media with ethanol (RU0; black), or
371 25uM RU-486 (RU25; blue) throughout adult life. Dashed black line denotes the median
372 survival. (B) 48-hour food consumption (uL) of 6 groups of 10 *TIGS > UAS-Slif^{anti}* females fed
373 liquid defined media (omitting cholesterol) for 48 hours, following 72h induction of
374 knockdown (RU25) or control (RU0) treatment. (C) Internal bacterial load of 15 and 45 day
375 old, and 45-day old Smurf (45S) *TIGS > UAS-Slif^{anti}* females, with and without induction of

intestinal *Slif* knockdown. Conventionally colonised mated females are used throughout. Bar graphs show mean and standard error. No significant difference by unpaired t-test. Box plots show the 25-75th percentiles and the median with the whiskers extending from min-max. Two-way Anova with Tukey's multiple comparisons P-values are shown below key in panel C.



Supplemental Figure 5: Intestinal *slimfast* levels regulate lifespan.

(A) Replicate lifespan of conventionally colonised mated *TIGS > UAS-slif*^{anti} females on defined media with ethanol (RU0; black), or 25uM Ru-486 (RU25; blue) throughout adult life. Dashed black line denotes the median survival. (B-E) Replicate lifespans of

387 conventionally colonised mated *TIGS > UAS-slif* females on defined media with ethanol
388 (RU0; black), 25uM Ru-486 throughout adult life (RU25; dark blue) or from day 35 (RU25
389 late, pale blue). Dashed black line denotes the median survival. (F) Internal bacterial load of
390 16 and 58 day old, and 58-day old Smurf flies (58S) mated female *TIGS > UAS-slif* females,
391 with induction of intestinal *slif* overexpression throughout adult lifespan (RU25) or from day
392 35 (Late RU25) and ethanol treated controls (RU0). Box plots show the 25-75th percentiles
393 and the median with the whiskers extending from min-max. Two-way Anova with Tukey's
394 multiple comparisons P-values are shown below graph in panel F.
395
396 To determine whether the age-related loss of *slif* expression is functionally relevant,
397 we knocked down *slimfast* expression specifically in intestinal tissue from early adulthood.
398 We used the *slif-antisense* line ($slif^{anti}$)[41] and the TIGS driver, a drug inducible geneswitch
399 Gal4[42,43], enabling us to restrict knockdown to the adult stage. We observed a significant
400 and reproducible lifespan extension in drug induced females compared to vehicle controls
401 (Fig. 5A and Supp. Fig 5A). Given that slimfast has been previously shown to regulate
402 feeding[44,45] and that reduced food intake itself can regulate lifespan we performed a
403 capillary feeding assay[34]. We found no observable difference in the volume of food
404 consumed (Fig. 5B). Considering that reduced intestinal *slif* may alter luminal nutrient
405 availability, subsequently altering the intestinal microbiota, we measured bacterial load
406 using qPCR of the bacterial 16S gene. Interestingly, we observed reduced bacterial load in
407 old $slif^{anti}$ females (Fig. 5C), both prior to and following loss of intestinal barrier function. In
408 contrast, overexpression of *slif* in the intestine for the full adult lifespan or during later adult
409 life had no consistent impact on lifespan and did not change intestinal bacterial load (Supp.
410 Fig. 5B-F). These data suggest loss of intestinal *slif* expression in later life may be protective,
411 perhaps by preventing uptake of excess amino acids contributed by the aged microbiota. In
412 addition, reduced *slif* expression may confer improved control of the intestinal microbiota,
413 perhaps reflecting improved overall health of the aged intestine in these flies.
414
415 **Discussion**
416 In this study we have developed a powerful new assay that enables us to investigate the
417 relationship between intestinal homeostasis and nutrient management in a genetically
418 tractable model system. We have used the D.U.M.P assay to demonstrate an increase in

419 amino acid levels in the faecal matter, and by proxy the intestinal lumen, of aged flies. It is
420 important to note that our targeted mass spectrometry approach measures only free amino
421 acids. Free amino acids in the faeces will include those remaining unabsorbed from the diet,
422 as well as those contributed by secretion from microbial or fly cells, and those released from
423 microbial or fly proteins by digestion. Comparison between the faecal content of
424 conventional and axenically reared flies has demonstrated the significant contribution of the
425 intestinal microbiota to the luminal amino acid pool. This finding is in line with previous
426 studies demonstrating the ability of the microbiota to rescue flies from undernutrition,
427 specifically low protein diets[46,47]. In fact, we find a higher contribution to amino acid load
428 from the intestinal microbiota than from the diet. These data also demonstrate the
429 significant changes in this contribution that result from age-related shifts in the microbial
430 population. The impact of changes in relative abundance of specific amino acids on health
431 and longevity warrents further investigation, but falls outside of the scope of this study.
432 In addition to the direct microbial contribution, secreted proteins and shed fly cells
433 may contribute to the nutrient pool. Elevated immune responses in the aged intestine could
434 raise both the levels of secreted proteins (including antimicrobial peptides and other
435 immune responsive proteins) and intestinal cell death and shedding[6,7,48,49]. These processes
436 are also driven by age-related changes to the intestinal microbiota. Therefore, the amino
437 acid increase recorded in the presence of the microbiota may not soley originate from
438 microbial cells. Reduced expression levels of a subset of amino acid transporters in the aged
439 intestine suggests that reduced amino acid transport into epithelial cells also contributes to
440 elevated luminal levels in aged animals. Our current data cannot distinguish between amino
441 acids remaining unabsorbed from the diet and those contributed from secreted fly proteins,
442 shed intestinal cells or microbial cells. It will be important to address the relative
443 contributions of these amino acid sources in future studies. However, that the percentage
444 contribution from the microbiota remains unchanged between young and old flies suggests
445 that changes occurring independently of the microbiota, such as reduced amino acid
446 transport, have minimal impact on the overall age-related increase in amino acid levels.
447 As we would expect from previous studies[32], increasing dietary amino acid levels
448 shortens lifespan. In contrast, intestinal knockdown of the amino acid transporter *slimfast*
449 extends lifespan. At first glance these data may seem contradictory, given that knockdown
450 of *slimfast* can be expected to raise luminal levels of the amino acids that it transports.

451 However, it is important to note that when feeding additional amino acids to young flies we
452 assume an increased uptake of amino acids by the intestine, and therefore elevated amino
453 acid levels in intestinal cells and other tissues. While reduced transporter expression may
454 result in some elevation of luminal amino acid levels it will also prevent cellular uptake of
455 excess amino acids and the resulting metabolic consequences. In effect reduced amino acid
456 transporter expression may decouple amino acid level in the intestinal lumen from that in
457 intestinal cells and reaching systemic circulation. Recent studies suggest elevated activity of
458 the amino acid sensing Target of Rapamycin (TOR) pathway, an established regulator of
459 longevity, may also have a role in intestinal decline. Recently, it has been demonstrated that
460 TOR inhibition delays age-related intestinal decline[4,50–53] with genetic interventions targeted
461 to intestinal stem cells sufficient to preserve homeostasis[4,53]. Evidence is also emerging that
462 elevated TOR activation in intestinal cells may be a feature of natural ageing[4,54]. The impact
463 of reduced *slimfast* levels on larval growth is mediated by reduced TOR activity[41], and we
464 suggest that reduced *slimfast* levels in the gut may protect against elevated luminal amino
465 acid levels via reduced TOR activity.
466 In this context it is also important to consider increased levels of individual amino
467 acids, such as the *slimfast* substrate arginine, may have a very different result to an increase
468 contributed by multiple amino acids. In fact, arginine has been shown to improve intestinal
469 immunity phenotypes in mice, through modulation of the microbiota[55]. Increasing arginine
470 levels also increases the diversity of murine microbiota[56,57] improving the phenotypes of a
471 colitis model, with benefits that are passed on through faecal transplantation and are
472 prevented by antibiotic treatment[56,58]. In addition to impacts on the microbiota, some
473 amino acids may have specific roles in intestinal health maintenance. For example,
474 increased glutamate concentration has been shown to stimulate intestinal stem cell (ISC)
475 proliferation in *Drosophila*[40] and in mice[59] and L-arginine has also shown to stimulate ISC
476 expansion in mice[60]. Beyond the gut arginine has been shown to have a positive association
477 with lifespan in a study of fibroblasts taken from a range of primate, avian and rodent
478 species[61] and its supplementation increases the lifespan of *C. elegans*[62]. However, arginine
479 supplementation has also been shown to increase the secretion of IGF-1[63] and growth
480 hormone[64], both of which have pro-ageing functions in mice[65–67], and to stimulate TOR
481 activity[68]. Increased arginine levels have been found in aged Drosophila[69] and
482 supplementation of 20mM arginine to the diet of Drosophila leads to a lifespan reduction,

483 and reduced fecundity[70]. These apparent contradictions highlight the complex pleiotropic
484 roles for individual amino acids acting in metabolism and as signalling molecules
485 systemically and within individual tissues.

486 Taken together our data suggest a model whereby age-related changes to the
487 microbiota elevate amino acid levels in the intestinal lumen while a concomitant reduction
488 in transporter expression protects the intestinal epithelium from the metabolic
489 consequences of excess amino acid uptake. This model has significant implications for the
490 rational design of anti-ageing nutritional therapies. For example, while past studies have
491 focused on the importance of dietary protein levels to healthy ageing (reviewed in
492 Babygirija and Lamming, 2021[32]), our data highlight additional sources of elevated amino
493 acid exposure in the aged intestine. Could reductions in dietary protein levels be effective in
494 improving health because they counter natural increases in amino acid exposure that result
495 from age-related intestinal decline? In addtion, there are currently calls to increase the daily
496 recommended protein intake for elderly persons to prevent or reduce age-related muscle
497 loss and sarcopenia[71]. Aged muscle cells are believed to be resistant to stimuli such that
498 elderly individuals must consume higher amounts of protein to stimulate muscle growth[72–
499 74]. However, our data suggest reduced amino acid uptake by the intestine may also underlie
500 reduced muscle mass in the elderly. Perhaps higher quantities of protein must be consumed
501 because a lower proportion is absorbed and bioavailable to the muscles. If this is the case,
502 an approach that focuses on maintaining intestinal health may delay the development of
503 sarcopenia and prevent additional risks to health resulting from high protein consumption.

504 In conclusion, our findings demonstrate that age-related intestinal decline
505 profoundly changes the local nutritional environment with consequences for age-related
506 health. While we have focused on one class of nutrient and on the impact of intestinal
507 ageing, the D.U.M.P. assay has much broader applicability. In combination with the genetic
508 tools available in *Drosophila* and the tractability of the *Drosophila* microbiota, the D.U.M.P.
509 assay will enable researchers in this field to assess the impact of a wide range of
510 perturbations on the gut nutrient pool. For example, how does nutrient availability change
511 during infection or following a loss of cellular homeostasis and subsequent regeneration?
512 What role does nutritional availability play in these processes? In what ways do specific
513 microbes contribute to the nutrient pool, and how does this change following perturbation
514 of the microbial community? Given that the local nutritional environment is not simply

515 determined by the diet, such studies are an essential complement to efforts to understand
516 the impact of the levels of specific nutrients on intestinal homeostasis, metabolic status, and
517 organismal health.
518
519 **Materials and Methods**
520
521 **Fly stocks**
522 This work used the wild-type Canton-S strain (gift of Linda Partridge), TIGS-2-GeneSwitch
523 driver line[75] (gift of David Walker), UAS-slif^{anti} (BDSC #52655) and UAS-slif (BDSC #52661).
524
525 **Fly culture**
526 Flies were maintained in either vials or bottles in incubators with 65% humidity and a 12-
527 hour light/dark cycle, at either 18°C or 25ºC. Stocks were maintained on cornmeal medium
528 comprised of 1 % agar (SLS, FLY1020), 3% dried inactivated yeast (Genesee Scientific, 62-
529 106), 1.9 % sucrose (Duchefa Biochemie, S0809), 3.8 % dextrose (Melford, G32040) and 9.1
530 % cornmeal (SLS, FLY1110), all given in wt/vol, and water at 90% of the final volume.
531 Preservatives; 10 % Nipagin (SLS, FLY1136) in ethanol and an acid mixture comprised of 4.15
532 % phosphoric acid (VWR, 20624.262) and 41.8 % propionic acid (Sigma, P5561) in water,
533 previously described by Lewis, 1960[76], were added following boiling.
534
535 **Defined media**
536 The standard defined medium was prepared as in Piper *et al*. 2014[29] using the suggested
537 addition of 50 % more vitamin solution and the exome matched amino acid concentrations
538 'FLYAA' reported in Piper *et al.* 2017[30]. Contrary to the published method, buffer A
539 containing the metal ion solutions, low solubility amino acids, cholesterol, MilliQ water and
540 acetate buffer was brought to a boil in the microwave, not autoclaved at 121 ° C for 15
541 minutes, except in the preparation of sterile medium for axenic flies. The remaining
542 ingredients of essential and non-essential amino acid solutions, cysteine and glutamic acid
543 solutions, vitamin solution, lipid-related metabolite solution, folate, 10 % nipagin in ethanol,
544 and propionic acid were added once buffer A was cooled to 65 ° C, and thoroughly
545 incorporated. Aged AA media was prepared in the same way, but both the quantities of
546 amino acids used to make the stock solutions and the volume of stock solution used was

547 altered. The masses of amino acids added to the AA media were calculated by the
548 difference between mean old and mean young concentrations measured by the D.U.M.P.
549 assay in conventionally reared flies, plus the original defined media amino acid
550 concentration for each amino acid. Complete recipes for both the standard and Aged AA
551 media are given in a supplemental protocol file attached to this manuscript.
552
553 **Lifespan**
554 All experiments were carried out using age-matched mated females, housed at a density of
555 27-32 flies per vial. These flies were flipped onto new food within 24 hours of eclosing. They
556 were then allowed at least a further 24 hours to mate before the mated females were
557 sorted under light CO_2 anaesthesia into vials containing defined media at 48-96 hours after
558 eclosion. Flies were flipped to fresh food every 2-3 days and the number of dead flies was
559 counted. At least 6 vials of 30 females were used per lifespan. To control for bacterial
560 growth on the food, control and experimental flies are always transferred to fresh food at
561 the same time points.
562 Mifepristone or RU-486 (Cayman chemicals, 10006317), hereafter referred to as RU,
563 food was prepared by the addition of 125 µL of 20 mg/ml RU stock solution per 100mL food.
564 To ensure sufficient volume for effective mixing 125 µL of RU stock was added to 125 µL of
565 100% ethanol per 100 mL of food prior to addition to the food. Control food was prepared
566 by the addition of 250 µL ethanol per 100 mL food. Food was mixed thoroughly to ensure a
567 homogenous solution.
568
569 **Smurf assay**
570 The smurf assay[31] for intestinal barrier integrity was performed by flipping flies onto food
571 containing 2.5 % FD&C blue food dye #1 for 24 hours (FastColours, 08BLU00201). After 24
572 hours, the flies were flipped back onto dye-free food and the number of 'smurf' and 'non-
573 smurf' flies were recorded.
574
575 **Generation of axenic and reassociated flies**
576 To generate sterile (axenic) flies, eggs were collected on apple juice agar plates seeded with
577 a paste of live bakers yeast and ddH_2O, then washed and treated with bleach and ethanol
578 as described previously[77]. All steps were carried out in a laminar flow hood. Briefly, <14

579 hour-old embryos were dechorionated in 2 % sodium hypochlorite solution for 2.5 minutes,
580 rinsed in 70 % ethanol for 5 minutes and then rinsed with ~200 mL sterile PBS. Embryos
581 were then resuspended in sterile 0.01 % PBSTx (PBS + 0.01 % Triton X-100 (Sigma, X100))
582 and transferred to sterile autoclaved cornmeal medium.
583 To re-associate sterilised embryos with parental gut bacteria, 100 µL adult fly
584 homogenate (1 fly/20 µL sterile PBS) was added to sterile cornmeal medium prior to
585 addition of axenic embryos and allowed to dry. Flies were surface sterilised for 5 minutes in
586 70 % ethanol prior to homogenisation to ensure only internal microbes were present in the
587 homogenate. Where conventionally reared flies were used as a further control to axenic or
588 re-associated flies, 30 mated females were placed in vials containing sterilised cornmeal
589 food and allowed to lay eggs for 24 hours, starting the evening prior to embryo collection.
590 All medium for sterile and control flies was sterilised by autoclaving for 15 minutes at 121
591 °C, and all equipment used to push flies was first sterilised in Virkon for at least 20 minutes,
592 then rinsed and stored in 80 % ethanol. Gas pads were subsequently dried by running CO_2
593 through them. To maintain sterility, axenic females were separated into lifespan vials next
594 to an open flame and flipped to new sterile food every 2-3 days in a laminar flow hood.
595 Sterile conditions were confirmed throughout lifespans by testing the sterility of
596 soiled vials. Soiled stoppers were pressed into single wells of 12-well plates (Starlab, E2996-
597 1610) containing 3 mL of pre-poured and set LB Miller Agar. The plates were incubated at 37
598 °C for 24-48 hours to check for bacterial growth.
599
600 **Drosophila Undigested Metabolite Profiling (D.U.M.P.) assay**
601
602 *Preparation*
603 This assay relies on knowing the full content of the defined media. To prepare, defined
604 media was cooked as described above, with the addition of 2.5 % blue food dye #1 after
605 cooking (as in the Smurf assay). As well as preparing 4 mL vials, the food was poured into
606 petri-dishes for the assembly of collection vials. Collection vials are open-ended glass tubes
607 with 2.4 cm external diameter and 9.6 cm high, similar dimensions to standard fly vials.
608 These were provided by the glassblowing workshop within the Durham University chemistry
609 department. Prior to beginning the assay, collection tubes were prepared by using each
610 tube to stamp out a disc of food from the prepared petri-dish. The food discs were secured

611 in place in the tube ends with parafilm. This plugs one end of the vial; the other end of the
612 tube was plugged with a foam Droso-Plug (SLS, FLY1000).
613 24 hours prior to sample collection, flies were flipped onto defined media containing 2.5 %
614 FD&C blue food dye #1. This ensures that the flies' guts will be full of the assay media
615 containing the dye for normalisation. Additionally, this is enough time for flies losing
616 intestinal barrier function to become clearly visible, enabling their exclusion from the assay.
617
618 *Sample collection*
619 To begin the assay, flies were sorted into the pre-prepared collection vials under light CO_2
620 anaesthesia. Approximately 55 - 65 non-smurf, mated females were sorted into each vial.
621 More flies do not increase the amount of faecal deposit as when overcrowded they get
622 stuck at the bottom of the vial. 6 collection vials were used per sample to ensure a large
623 enough sample for full analysis. Once the flies awoke from anaesthesia, they were
624 incubated at 25°C for between 4 and 5 hours to eat and defecate as usual. Standing the vials
625 at a 45° angle results in an improved sample, particularly when collecting samples from aged
626 flies with reduced mobility. Following incubation, the flies were anaesthetised with CO_2, the
627 parafilm removed and the food disc along with the flies was pushed out from the bottom of
628 the tube. The outside of the vial and the food residue was cleaned with a tissue dampened
629 by milliQ water until the tissue came away clean.
630 The collection vials were all the same weight (26.91g ± 0.41) and fit inside 50mL
631 falcon tubes. Once within the 50 mL tube, the collection vials were thoroughly rinsed with
632 1.5 mL of water to collect the polar, water-soluble faecal fraction, and spun down. The same
633 1.5 mL of water was used to rinse each tube. This ensured the sample could be frozen and
634 lyophilised quickly. Two falcon tubes are used, with the collection vials each being washed,
635 then the water sample split between the falcons, to ensure the tubes are balanced for
636 centrifugation. Once all tubes are rinsed, the sample is transferred to a microcentrifuge
637 tube. The rinse is repeated a second time to ensure all faecal matter is collected, resulting in
638 two water-wash samples of 1.5 mL.
639 The vials were then rinsed thoroughly with a 1:1 mixture of DCM and methanol to
640 collect non-polar deposits. The rinses were carried out in a small glass beaker to collect the
641 sample. The volume for this wash was not limited, but approximately 5 mL was used for all

642 the tubes. Plastic use should be avoided with the DCM:MeOH wash. All samples were frozen
643 at -80 ° C prior to downstream processing.
644
645 *Sample processing*
646 The two water washes were combined, and 1-2 μL was removed and diluted 1/100 for the
647 blue dye to be quantified at 628nm absorbance, on the Bio-tek Synergy H4, alongside a
648 standard curve of known concentration. The remaining sample was split three ways to
649 enable analyte-specific processing and quantification. While we only quantified amino acids
650 in our final samples for the purpose of this study, we provide details for initial processing of
651 the other sample types here. The amino acid and sugar fractions were lyophilised and
652 weighed, then resuspended in 40 μL 0.1N HCl, or 72:28 MeCN:H_2O + 0.1% NH_4CN per mg
653 respectively. The resuspended amino acid sample was diluted 1:100 in HILIC A for LC-MS/MS
654 analysis, and the sugar samples were diluted between 1:10 and 1:100 in the 72:28
655 MeCN:H_2O + 0.1% NH_4CN prior to LC-MS/MS analysis. The third fraction was for cholesterol
656 analysis. An equal volume of DCM was added to the water fraction, then the sample was
657 vortexed for 2 minutes prior to 15 minutes of centrifugation at 1000x rpm. The DCM layer
658 was removed and added to the DCM:MeOH wash, this was dried under a stream of N_2, then
659 resuspended in 200 μL 1:1 IPA:MeCN, and spiked with 10 μM d6-cholesterol prior to LC-
660 MS/MS analysis.
661
662 *Amino acid quantitation*
663 LC-MS/MS analysis was performed on a Shimadzu Nexera X2 Ultra-Fast Liquid
664 Chromatography system consisting of binary pumps, an on-line degassing unit, an
665 autosampler, and a column oven (Shimadzu Corporation, Kyoto, Japan), coupled with an AB
666 Sciex 6500 QTRAP mass spectrometer consisting of an electrospray ionization (ESI) source
667 (AB SCIEX, Framingham, MA, USA). 1uL of sample of injected on to a BEH Amide (1.0 x
668 100mm, Waters, Milford, MA, USA), BEH HILIC (1.0 x 100mm, Waters, Milford, MA, USA) or
669 Ascentis HILIC (2.1 x 150mm, Waters, Milford, MA, USA) maintained at 40 °C, at a flow rate
670 of 0.2 mL/min or 0.3mL/min for the 1.0 and 2.1mm columns respectively. Three columns
671 were used to enable separation of isomers leucine and isoleucine, and optimal peak shape
672 for more accurate quantification. The mobile phases consisted of Solution A (85% MeCN +
673 0.15% FA + 10mM ammonium formate) and Solution B (85% H_2) + 15% MeCN + 0.15% FA +

674 10mM ammonium formate). A gradient elution was optimized for the separation of amino
675 acids with 1.5 min equilibration 0-6.0 min, 0% Solution B; 6.0-10 min, 0-100% Solution B; 10-
676 12 min, 100% Solution B; 12-12.1 min, 0% Solution B; 12.1-18 min, 0% Solution B; with a
677 total run time of 21 min. The ion source was operated in positive mode under an optimal
678 condition: curtain gas, 40 psi; nebulizer gas, 50 psi; auxiliary gas, 50 psi; ion spray voltage,
679 5500 V; and temperature, 500°C. Optimal multiple-reaction monitoring (MRM) transitions
680 were identified for the amino acids (Supplemental table 2). Quantification was performed
681 using an external calibration curve, amino acids stocks in 0.1N HCl were diluted with HILIC A
682 as per sample preparation. Data acquisition and analysis were all performed with Analyst
683 1.6.3 software (AB SCIEX)
684
685 *Analysis*
686 Relative quantification was performed using the blue food dye; the mass-spec calculated
687 concentration (μM) was adjusted for the sample dilution. This was then divided by the
688 concentration of the blue dye (μM) for that sample.
689 The percentage change between young and old timepoints was calculated using the
690 equation:
691 $\% \, change = \frac{O - Y}{Y} * 100$
692 Where O is the normalised concentration at the old timepoint, and Y is the normalised
693 concentration at the young timepoint. The young concentrations from all replicates were
694 compared against the old concentrations from each replicate.
695 The microbial concentration was calculated at young and old timepoints by:
696 $Microbial\ contribution = conventional\ concentration - axenic\ concentration$
697 Where the conventional concentrations from each replicate were compared against the
698 axenic concentrations for each replicate and at every timepoint.
699
700
701
702
703
704

705 **Supplemental table 2.** MRM transitions for the amino acids quantified

Q1 m/z	Q3 m/z	Time (msec)	Amino Acid	DP (V)	EP (V)	CE (V)	CXP (V)
166.1	120.1	50	Phe 1	30	10	15	12
132.1	86.1	50	Leu 1	36	10	23	14
132.1	86.1	50	Ile 1	40	7	15	8
150	104	50	Met 1	30	10	15	12
118.1	72.1	50	Val 1	30	10	15	12
116	70.1	50	Pro 1	30	10	15	12
182.1	136.1	50	Tyr 1	30	10	15	12
90.1	44.1	50	Ala 1	30	10	10	12
120	74.1	50	Thr 1	30	10	15	12
76	30.1	50	Gly 1	46	10	20	12
106	60	50	Ser 1	30	10	10	12
148	84	50	Glu 1	30	10	15	12
134	88	50	Asp 1	26	10	13	14
156	110.1	50	His 1	30	10	15	12
175.1	70.1	50	Arg 1	30	10	15	12
147.1	84.1	50	Lys 1	30	10	15	12
166	103	50	Phe 2	31	10	35	28
132.1	69	50	Leu 2	36	10	23	20
150	133	50	Met 2	21	10	13	8
118	55	50	Val 2	36	10	29	6
182.1	165	50	Tyr 2	46	10	19	6
120	56	50	Thr 2	86	10	23	6
106	42	50	Ser 2	31	10	31	4
148	102	50	Glu 2	21	10	15	4
134	70	50	Asp 2	36	10	25	6
156	93	50	His 2	41	10	31	8
175	116	50	Arg 2	46	10	19	6
147	130	50	Lys 2	16	10	13	6
122	76	50	Cys 1	30	10	10	15
205.1	146.1	50	Trp 1	36	10	10	23
147.1	84.1	50	Gln 1	30	10	10	15
133	74	50	Asn 1	30	10	25	25
133	116	50	Asn 2	31	10	13	8
205.1	188	50	Trp 2	41	7	15	9
147	102	50	Gln 2	31	10	19	12
132.1	69	50	Ile 2	40	7	24	9

706
707

708 **Capillary feeding (CAFÉ) assay**

709 Food consumption was measured, essentially as previously described[34], using liquid defined
710 media made without agar and, due to issues in solubility, without cholesterol. Cotton wool
711 balls were placed at the bottom of drosophila vials and saturated with water. Any excess
712 water was removed, and the vial walls were dried. 10 μL pipette tips were trimmed to
713 enable capillary tubes to extend from the end with a tight fit so they wouldn't slip.
714 Drosophila flugs were cut in half laterally, and three prepared tips were inserted through
715 the flug. The capillary tubes were prepared by loading with 2 μL of mineral oil to limit
716 evaporation, and 10 μL of liquid food, then the interface between oil and food was marked.
717 10 mated female flies were sorted into the prepared vials. Each condition had a
718 minimum of 10 vials. Additionally, a minimum of three evaporation control vials, containing
719 no flies, were used for each experiment. Once the flies were awake within the vial, the
720 capillary tubes were inserted into the tips, so the ends extended just beyond the base of the
721 tip. The capillary tubes were changed every 24 hours, and the distance between the
722 interface between oil and food, and the mark denoting the starting interface was recorded.
723 The flies were allowed 24 hours to acclimate to the new feeding setup prior to
724 measurements being recorded. Following acclimation, the assay was performed for 24-48
725 hours. Food consumption was calculated in mm, by calculating the difference in the start
726 and end interface and subtracting the average difference in the start and end interface of
727 the evaporation controls. A conversion factor (X μL/mm) was calculated for each capillary
728 tube by dividing 10 (the amount of liquid food), by the starting interface (mm). This was
729 then used to convert food consumption in mm to μL.

730

731 **Bacterial DNA extraction**

732 30 whole flies were sorted under light CO_2 anaesthesia into microcentrifuge tubes and
733 frozen at -80 °C. Flies were then surface sterilised in 70 % ethanol for 5 minutes and rinsed
734 twice in sterile PBS. 5 flies were sorted into sterile microcentrifuge tubes and homogenised
735 in 200 µl lysis buffer (1 X TE, 1 % Triton X-100, 1/100 proteinase K (NEB, P8107S)) using a
736 mechanical pestle, with a further 300 µl of lysis buffer being added after homogenisation.
737 Lysate was incubated for 1.5 hours at 55 °C then transferred to screw cap vials containing
738 0.4 g sterilised low binding 100-micron silica beads (OPS Diagnostics, BLBG 100-200-11) and

739 vortexed for 10 minutes at max speed. Lysate was then transferred back to the original tube
740 and incubated for a further 1.5 hours at 55 °C followed by 10 minutes at 95 °C.
741
742 **RNA extraction**
743 Total RNA was extracted from 5 midguts per biological replicate. Midguts were dissected in
744 ice-cold PBS and included the cardia and midgut, through to the midgut-hindgut junction,
745 excluding the hindgut and Malpighian tubules. Midguts were homogenised in 100 µL
746 TRIzol™ (Thermofisher, 15596026) using a motorised pestle. RNA was extracted following
747 the standard TRIzol protocol.
748
749 **cDNA preparation**
750 For a 10 µL reaction, 1 µL DNase buffer (Thermo, EN0521) and 0.5 µL DNase 1 (Thermo,
751 EN0521) were added to 8.5 µL RNA solution and incubated for 30 minutes at 37 °C, to
752 remove any DNA contaminants. The DNase enzyme was then deactivated by the addition of
753 0.5 µL 50mM EDTA (Thermo, EN0521) and subsequent incubation for 10 minutes at 65 °C.
754 cDNA synthesis was carried out using Thermo Scientific's First Strand cDNA Synthesis kit. In
755 short, 1 µL of random hexamers (Thermo, SO142) were added and the samples were
756 incubated at 70 °C for 5 minutes before a 9 µL of a master mix containing 4 µL first strand
757 buffer (Thermo, EPO442), 0.5 µL RIbolock RNase inhibitor (Thermo, EO0382), 2 µL 10mM
758 dNTPs (Thermo, R0181), 0.2 µL RevertAid reverse transcriptase (Thermo, EPO442) and 2.3
759 µL molecular grade water (Sigma, W4502) per well, was added. The samples were then
760 incubated for 10 minutes at room temperature, followed by 1 hour at 37 °C and 10 minutes
761 at 70 °C. The samples were spun following each incubation step.
762
763 **Quantitative PCR**
764 qPCR was performed with Power SYBR™ Green master mix (Fisher, 10219284) on a Bio-RAD
765 CFX Connect Real-Time PCR detection system. Either 4.5 µL bacterial DNA or 1 µL of
766 *Drosophila* cDNA sample was used in a 10 µL reaction with 5 µL of Power SYBR™ Green and
767 0.5 µL of L + R primer mix. Any remaining volume was made up with molecular grade water.
768 Cycling conditions were: 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds followed
769 by 60°C for 60 seconds. All calculated gene expression values were normalised to the value

770 of the housekeeping gene rp49. Levels of the bacterial 16S rRNA gene were normalised to
771 the level of the *Drosophila* Actin gene. The primer sequences used are given in table 1.
772
773 **Table 1:** Primer sequences

Target	Forward primer	Reverse primer	Reference
Actin 5C	TTGTCTGGGCAAGAGGATCAG	ACCACTCGCACTTGCACTTTC	[7]
CG1698	CCAGGCGTATGTGGGTTCATT	AGAGAGCGTCTTCTTGCCATT	This study
CG43693	CCGTTGGTAATCTCTCGGAAAA	AGCGATCCCTTTAGCAAGTGT	This study
CG5535	TTATCCGCCTTCGATCTTACCG	CGGCATATTGCTTGGACACTT	This study
CG7255	CGGCAGTGTCTCAAAGTCG	TGGCGAACCATTGTTCAGTAT	This study
CG8785	AATGACTTCAACTCGAAAGCCA	GCTCGAAGGGATGGTAGGGA	This study
DmGlut	CGCCAATTAGTACCGAGATGA	CGCTGGGGTATCACGAAAT	This study
Eaat1	ATTGTGCCGCTTTTGGTCTCA	GTAATGGCTCTGGTAGCAATCTT	This study
Kazachoc (kcc)	GCCGTGGAAATCGTATTGAC	AGCGTCCTTGGTGAAGTCC	This study
NAAT1	CTTATGGGTTTGGTCTACGTTACA	CAGACCATAAACCCACACGA	This study
Minidiscs (mnd)	TGTGCCCTCGCTGATATTC	CCACGTAGCTGACGTAGTTGA	This study
Pathetic (path)	ATGTGCTCCGGTCTCATCAT	CTTGTGTCCGCATTTTACCA	This study
Rp49	ATCGGTTACGGATCGAACAA	GACAATCTCCTTGCGCTTCT	This study
Slimfast (slif)	CACAGTGAACAGCTACACGAAGA	GGTCCAGGTTAAAGAGCATGG	This study
Sobremesa	TGGGGTGGCTTTAATTGTTGG	CAGTGCGAACCAGTAAACCG	This study
16S	AGAGTTTGATCCTGGCTCAG	CTGCTGCCTYCCGTA	[78]

774
775
776 **Statistics**
777 All Statistical analyses were performed using GraphPad Prism 9, excluding the binomial tests
778 and principal component analyses which were performed in R (Rstudio build 372, R version
779 4. 0. 3). Survival curve analysis was completed using the log-rank test in GraphPad Prism;
780 the median survival was calculated by Graph Pad Prism 8 and is an approximation to the
781 closest sampling time. Quantitative PCR data and normalised DUMP amino acid data were
782 cleaned in GraphPad Prism, with outliers detected using the Robust regression and outlier
783 removal (ROUT) method with Q=1. Normality of the cleaned data was tested by Shapiro-
784 Wilk tests to determine the most appropriate statistical test. Differences between two
785 points were analysed by students t-test, or mann-whitney tests for normally and non-
786 normally distributed data respectively. Differences in gene expression values between more
787 than two time points, within or between conditions, were analysed by *two way Anova with*
788 *Tukeys multiple comparison.* P-values less than 0.05 were considered statistically significant.
789 Bar-graph error bars depict ± standard error of the mean (SEM). For all box-plots shown,

790 boxes display the 25-75th percentiles, with the horizontal bar at the median, and whiskers
791 extending from the minimum to maximum points.
792
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800
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