1 **Title:** Genetic variance in the murine defensin locus modulates glucose homeostasis

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22 **Competing interests**: the authors declare no competing interests

Abstract

Insulin resistance is a heritable risk factor for many chronic diseases; however, the genetic drivers remain elusive. In seeking these, we performed genetic mapping of insulin sensitivity in 670 chow-fed Diversity Outbred in Australia (DOz) mice and identified a genome-wide significant locus (QTL) on chromosome 8 encompassing 17 defensin genes. By taking a systems genetics approach, we ultimately identified alpha-defensin 26 (Defa26) as the causal gene in this region. To validate these findings, we synthesized Defa26 and performed diet supplementation experiments in two mouse strains with distinct endogenous Defa26 expression levels. In the strain with relatively lower endogenous expression (C57BL/6J) supplementation improved insulin sensitivity and reduced gut permeability, while in the strain with higher endogenous expression (A/J) it caused hypoinsulinemia, glucose intolerance and muscle wasting. Based on gut microbiome and plasma bile acid profiling this appeared to be the result of disrupted microbial bile acid metabolism. These data illustrate the danger of single strain over-reliance and provide the first evidence of a link between host-genetics and insulin sensitivity which is mediated by the microbiome.

39 **Introduction**

40 Insulin is among the most potent hormones in the human body; therefore, it is not
41 surprising that defects in insulin action, such as insulin resistance (IR) are shared risk
42 factors for many chronic diseases (1). Studies in twins and first-degree relatives of
43 individuals with Type 2 Diabetes have provided strong evidence of a genetic
44 component (2, 3). However, with some notable exceptions (4-6), genome-wide
45 association studies have failed to identify loci for IR. One potential explanation is that
46 the diversity of human environments confounds genetic signals via complex gene-by-
47 environment interactions. One manifestation of the environment that has been
48 implicated in metabolic disease is the gut microbiome (7-9). Intriguingly, some
49 microbial compositions have been shown to have beneficial effects on metabolic
50 health while others appear harmful (10). These effects are thought to be conveyed via
51 diverse signalling molecules including peptides (11, 12), metabolites (13, 14) and bile
52 acids (15-18). However, the host-microbiome relationship is bi-directional, as host
53 genetics can also influence gut microbial composition. Compelling evidence of this has
54 been provided by human genome-wide association and twin studies (19, 20).
55 Furthermore, a recent study in mice revealed that one quarter of all detected microbial
56 taxa have significant quantitative trait loci (QTL) implying host regulation of their
57 abundance (21). Notably, this includes the metabolically beneficial *Akkermansia*
58 *muciniphila*. However, it is unknown if genetic drivers of microbe abundance can
59 modulate insulin sensitivity.

60

61 One mechanism for host regulation of the gut microbiota is the secretion of anti-
62 microbial peptides called defensins into the intestinal lumen. Defensins are an ancient

63 component of the immune system, found across the tree-of-life from plants to humans.
64 They are small peptides (29-40 amino acids) with potent antibacterial and antiviral
65 activities (22). In humans, α-defensins are produced by neutrophils and specialised
66 gut epithelial cells, called Paneth cells. However, in mice, Paneth cells are the only
67 source of α-defensins (also called cryptidins) (23). Defensin secretion is regulated in
68 response to bacteria, nutrients or cholinergic agonists (24). Genetic variation in α-
69 defensin expression among different mouse strains has been reported (25) although
70 this has not been systematically examined. Defensins have also previously been
71 linked to metabolic health. Oral administration of human α-defensin-5 to mice
72 ameliorated liver fibrosis, diet-induced non-alcoholic liver steatosis, dyslipidaemia and
73 glucose intolerance (26-29). There has been some interest in defensins as a
74 therapeutic due to their oral bioavailability, a characteristic lacking in most peptide-
75 based therapies.

76

77 Mice are a valuable tool for studying human diseases due to their genetic and
78 physiologic similarities. Moreover, they afford the precise environmental control
79 required to detect genetic loci associated with complex diseases. This has been
80 illustrated by studies in panels of inbred mouse strains fed different diets (30-34) and
81 genetically diverse mouse populations such as Jackson Laboratory's Diversity
82 Outbred (DO) mice (35-38). We have established a similar population of mice, which
83 we term Diversity Outbred in Australia (DOz), and have previously used this population
84 to investigate skeletal muscle insulin resistance (39), and the metabolic consequences
85 of weight-cycling (40). Here, we use these mice to explore genetic drivers of insulin
86 sensitivity. Using the Matsuda Index as a surrogate measure of whole-body insulin

87 sensitivity, we identify a QTL within the defensin locus. Sequence variation at this
88 locus were associated with increased expression of α-defensin 26 (Defa26) and the
89 abundance of metabolically beneficial microbes. We validated this observation via
90 dietary supplementation with synthetic Defa26 in C57BL/6J mice, before going on to
91 demonstrate maladaptive effects in another strain, potentially via changes in microbial
92 derived bile acids. These data provide insight into the genetic architecture of insulin
93 sensitivity and highlight the limitations of testing therapeutics in single mouse strains.

94 **Results**

95 *Whole body insulin sensitivity is genetically linked to the defensin locus on*
96 *chromosome 8*

97 To quantify whole body insulin sensitivity, we conducted glucose tolerance tests on
98 670 chow-fed male DOz mice (Figure 1A) and used these data to calculate the
99 Matsuda Index, a surrogate measure of insulin sensitivity (41). Consistent with our
100 previous work (39), we observed profound variation in the Matsuda Index despite all
101 animals being housed under identical conditions and fed the same chow diet. We next
102 performed genetic mapping of the Matsuda Index (Figure 1B) and identified 2 genome-
103 wide significant quantitative trait loci (QTL), one on chromosome 2 at 162.2 Mbp and
104 a second on chromosome 8 at 21.7 MBp. The chromosome 8 locus centred over the
105 defensin gene cluster, a syntenic region shared between mice and humans (42), which
106 contains 53 defensin genes and 22 defensin pseudogenes (Figure 1C). Considering
107 previous links between defensins and metabolic disease (26-29) we selected this QTL
108 for further validation.

109

110 To stratify mice for further analysis we conducted single nucleotide polymorphism
111 (SNP) analysis of the defensin locus. We identified one intergenic SNP rs231642830,
112 and one structural variant SV_8_21749161_21749163, with genome-wide significant
113 logarithm of the odds (LOD) scores (Figure 1D). We categorised mice by the number
114 of minor alleles they carried at the rs231642830 SNP (AA = no minor alleles, AB = one
115 copy, BB = two copies). Animals carrying the AB allele had identical glucose tolerance
116 to 'control' AA mice despite a 50% reduction in insulin at the 15-minute time point
117 (Figure 1E,F) and increased insulin sensitivity determined by the Matsuda Index
118 (Figure 1G). These data suggest genetic variance at the defensin locus is linked to
119 improved whole body insulin sensitivity.

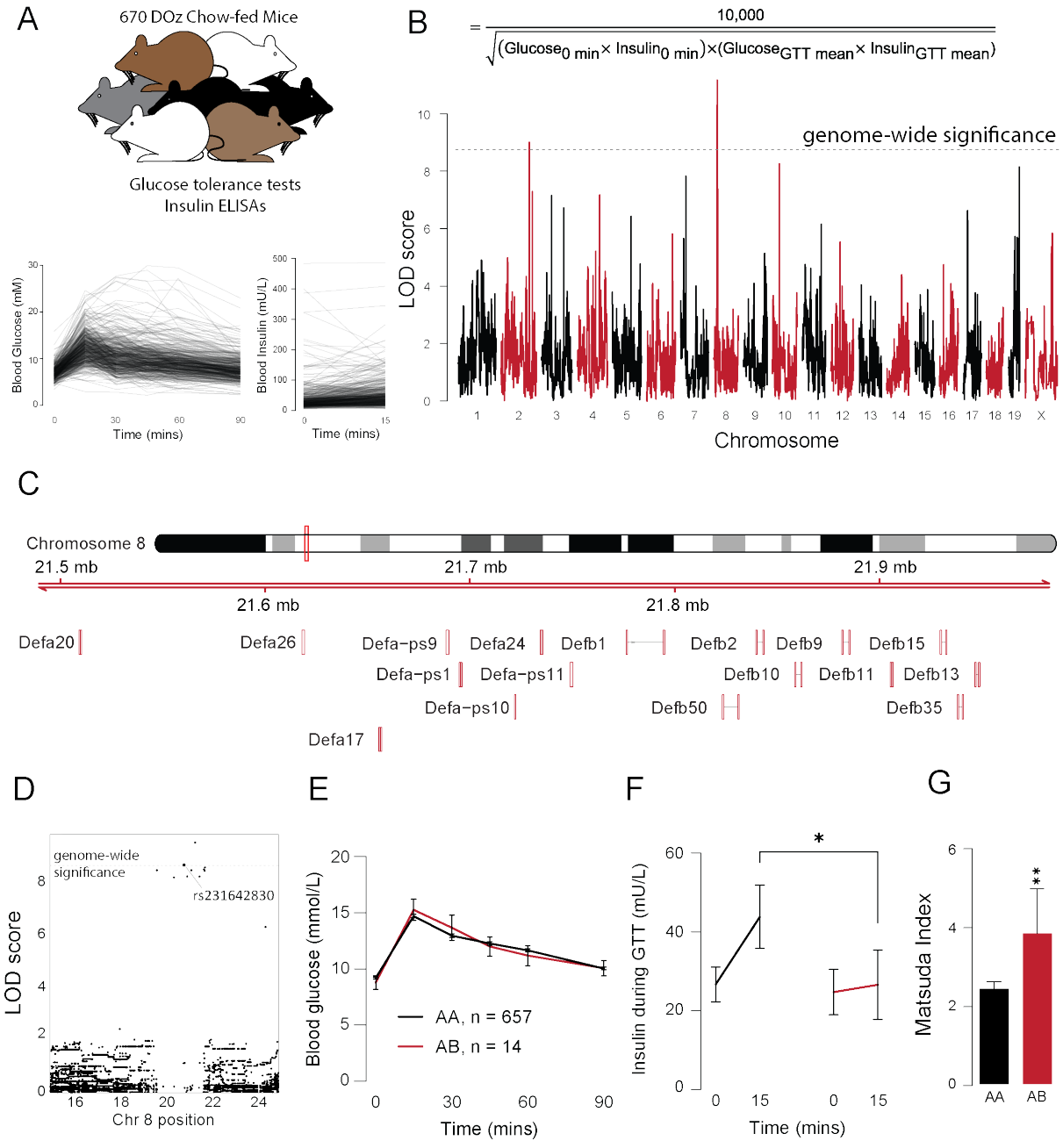


Figure 1 – Genetic mapping of insulin sensitivity in 670 chow-fed Diversity Outbred in Australia mice. A) Glucose and insulin concentrations during a glucose tolerance test in 670 DOz mice. **B)** The equation used to calculate the Matsuda Index and genetic mapping of the Matsuda Index in DOz mice. **C)** The defensin locus on chromosome 8. **D)** Single nucleotide polymorphism (SNP) mapping of the Matsuda Index on chromosome 8. **E)** Blood glucose concentrations during a glucose tolerance test of mice carrying the minor allele of rs231642830 (AB) and non-carrier (AA) controls. **F)** Blood insulin concentrations during a glucose tolerance test of mice carrying the minor allele of rs231642830 (AB) and non-carrier (AA) controls. **G)** Matsuda Index of mice carrying the minor allele of rs231642830 (AB)

129 and non-carrier (AA) controls. Data are mean with biological replicates shown as individual data points
130 or noted in figure, in F) and G) error bars represent S.E.M. * P < 0.05 compared to AA mice.

131

132 *A single nucleotide polymorphism within the defensin locus associates with insulin*
133 *sensitivity and Akkermansia muciniphila abundance*

134 In mice, defensins are secreted from Paneth cells in the intestinal crypt into the gut
135 lumen to modulate microbial composition (Figure 2A). Therefore, we performed 16S
136 rRNA sequencing to profile the microbiome composition of caecal contents of mice
137 from three groups: 1) mice carrying the putative protective rs231642830 allele (AB),
138 2) cage mates of these mice, and 3) a subset of AA control mice (Figure 2B). Because
139 mice are coprophagic, analysing cage mates allows us to test for co-housing effects
140 on insulin sensitivity, evidence for which would support a microbiome mediated
141 mechanism of action.

142

143 All AB mice had been separately housed, and AA control mice were chosen at random
144 from across 7 different cages that had no AB mice. No significant difference in alpha
145 diversity (Inverse Simpson) was observed between groups (Figure 2C). However,
146 beta-diversity analysis (Bray-Curtis dissimilarity and PERMANOVA) (Figure 2D) of the
147 three groups revealed a trend towards differences in overall microbiome composition
148 between AB mice and AA controls (p value = 0.05), and between AA controls and cage
149 mates of AB mice (p value = 0.055), but not between AB mice and their cage mates
150 (p value = 0.622). Analysis of the combined groups (AB mice + cage mates), against
151 the AA control mice revealed divergent microbiomes (p value = 0.009).

152

153 Differential abundance analysis of these groups by Analysis of Compositions of
154 Microbiomes with Bias Correction (ANCOM-BC) revealed that compared to AA
155 controls, *Akkermansia muciniphila* (Figure 2E, F), *Bifidobacterium pseudolongum*
156 (Figure 2E, G) and *Ligilactobacillus spp.* (Figure 2E, H), were higher in cage mate and
157 AB mice, while *Limosilactobacilius spp*. (Figure 2E, I) were lower. Intriguingly, *A.*
158 *muciniphila* has strong links to metabolic health and has been shown to increase in
159 response to α-defensin administration in C57BL/6J mice (11, 12, 29, 43, 44). These
160 data are consistent with AB mice possessing altered microbiomes relative to AA mice,
161 and this can be transmitted to their cage mates. Critically, cage mates of AB mice also
162 trended towards greater insulin sensitivity relative to AA control mice (Figure 2J)
163 suggesting that insulin sensitivity differences between AA and AB mice may be
164 transferable by cohousing via coprophagy.

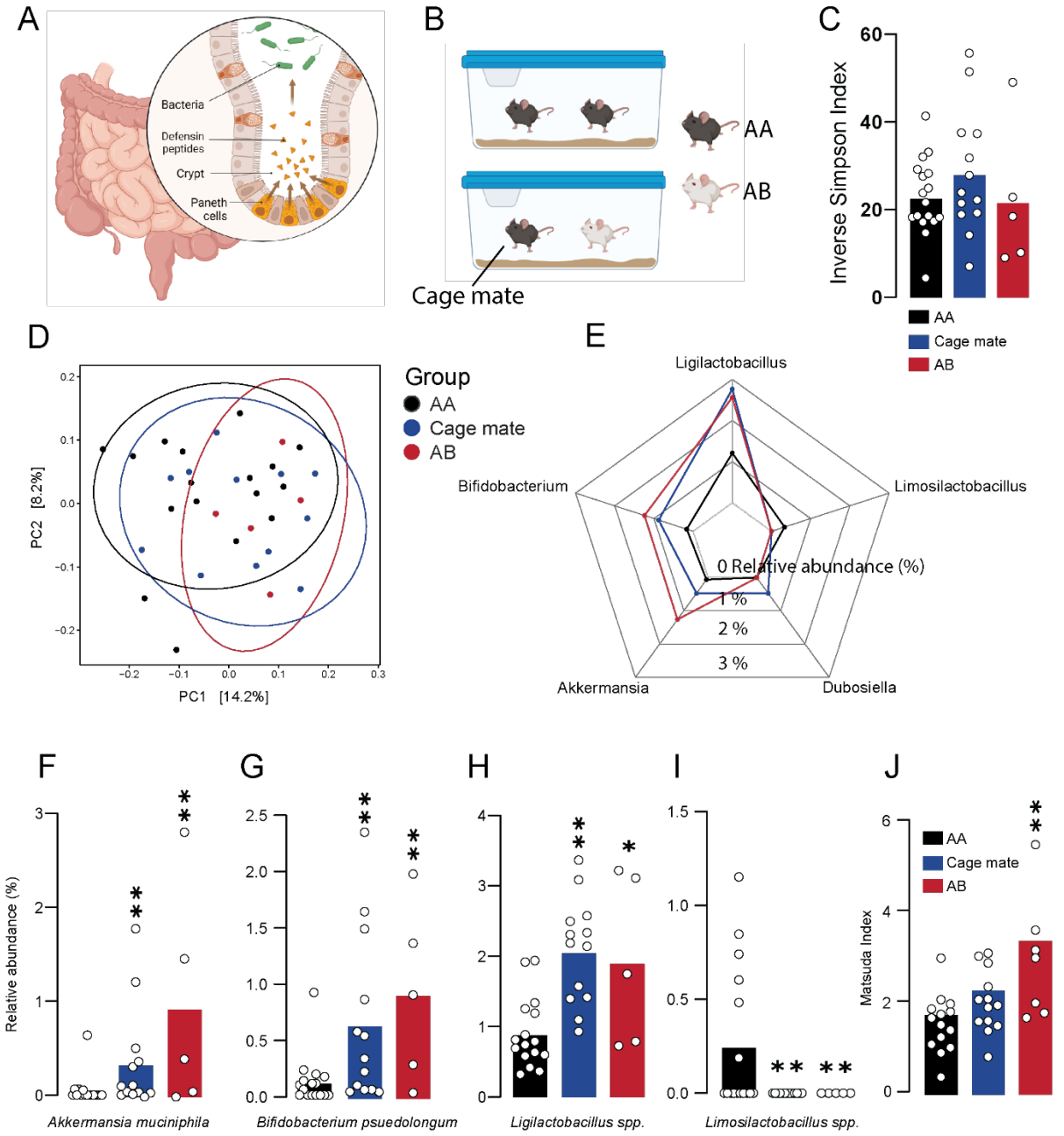


Figure 2 – Analysis of microbial composition and co-housing effects in mice carrying a putative insulin sensitivity allele within the defensin locus. A) Schematic of murine defensin secretion from Paneth cells into the small intestine. **B)** Schematic of the study design including mice carrying rs231642830 (AB), non-carrier controls (AA), and the cage mates of mice carrying rs231642830 (Cage mate). **C)** Alpha diversity (Inverse Simpson) of AB, AA and Cage mates. **D)** Visualisation of beta-diversity between AB, AA and Cage mate mice calculated by Bray-Curtis dissimilarity. **E)** Relative abundances of differentially represented microbes between AB, AA and Cage mate mice. **F)** Relative abundance of *Akkermansia muciniphila* in AB, AA and Cage mate mice. **G)** Relative abundance of

174 *Bifidobacterium psuedolongum* in AB, AA and Cage mate mice. **H)** Relative abundance of
175 *Ligilactobacillus spp*. in AB, AA and Cage mate mice. **I)** Relative abundance of *Limosilactobacillus spp*.
176 in AB, AA and Cage mate mice. **J)** Matsuda Index in AB, AA and Cage mate mice. Data are mean with
177 biological replicates shown as individual data points. ** P < 0.01, * P < 0.05 compared to AA mice.

178

179 *Alpha defensin 26 positively correlates with insulin sensitivity and with founder strain*
180 *contributions towards the Matsuda QTL in the defensin locus.*
181 We next sought to identify the specific defensin isoform that confers increased insulin
182 sensitivity. To do this we quantified small intestine defensin isoform protein expression
183 and insulin sensitivity in the Diversity Outbred founder strains (Figure 3A). One
184 advantage of genetic analyses in DOz is that the QTL analysis also provides the
185 contribution of each founder strain towards the QTL signal, and this allows validation
186 experiments to be conducted in the founder strains. Consistent with our previous work
187 (30) and that of others (33, 34), we observed significant variation in glucose tolerance
188 (Figure S1A-H), insulin sensitivity, and adiposity between the inbred founder strains
189 (Figure 3B-C). Using liquid chromatography coupled to tandem mass spectrometry we
190 detected 9 distinct defensin isoforms across 7 strains. To rule out differences in Paneth
191 cell abundance (25) influencing the apparent defensin expression levels, we
192 normalised defensin peptide expression to the average of all defensins within each
193 strain. Correlation analysis of normalised defensin isoform expression revealed that a
194 single isoform, α-defensin 26 (Defa26), was positively correlated with insulin sensitivity
195 (R = 0.73, P < 0.05; Figure 3D,E). We then calculated the QTL founder effects for
196 Matsuda Index on the chromosome 8 locus: A/J, NOD/ShiLtJ, and to a lesser extent
197 129S1/SvImJ, contributed positively while CAST/EiJ, NZO/HILtJ, WSB/EiJ, C57BL/6J
198 and PWK/PhJ contributed negatively towards the QTL (Figure 3F). Correlating the

founder effects at 21.7 MBp on chromosome 8 with mean normalised defensin levels revealed that only alpha-defensin 26 levels varied in accordance with the contributions of each founder strain to the QTL (R = 0.89, P < 0.01; Figure 3G).

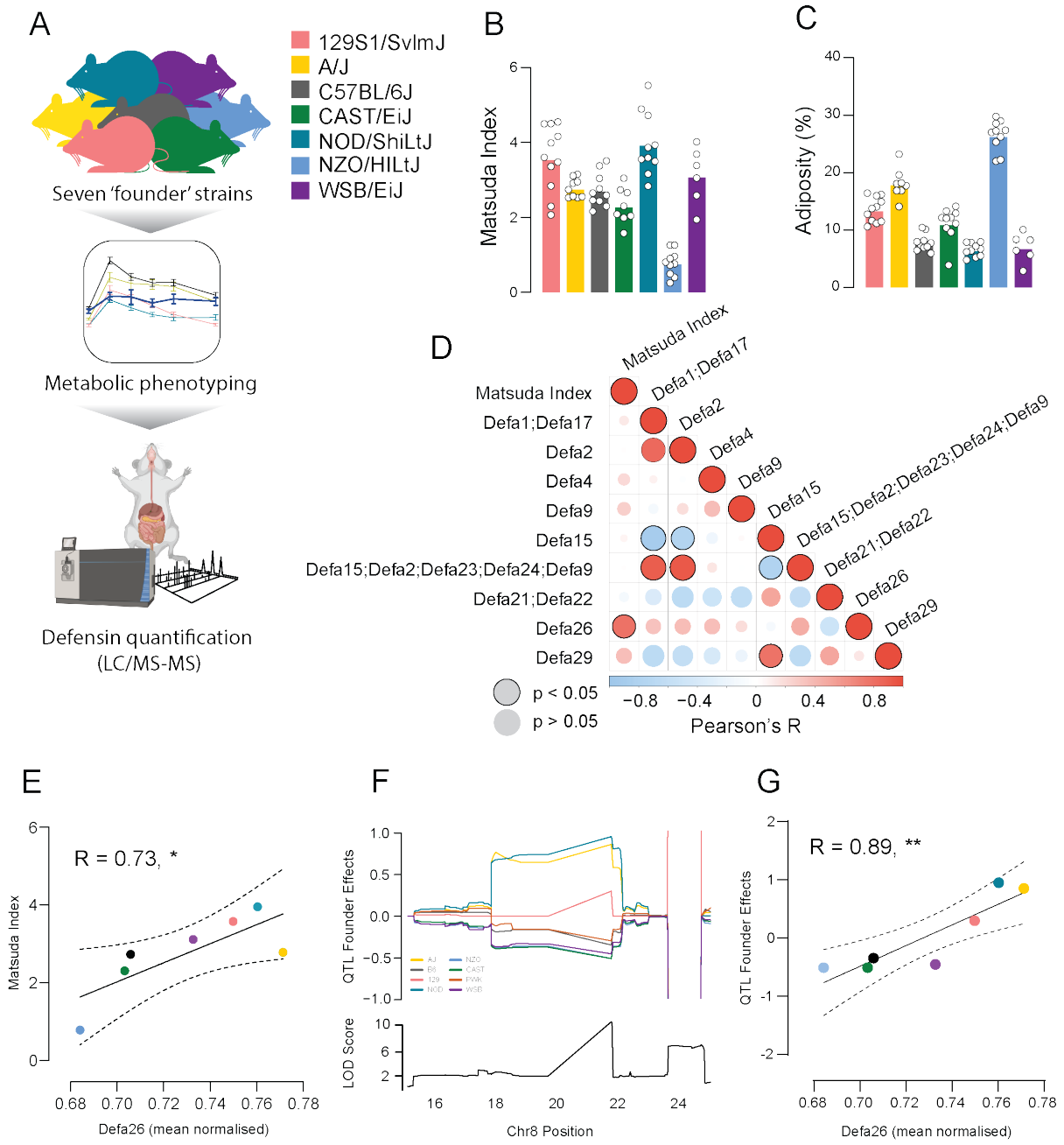


Figure 3 – Analysis of defensin protein expression in Diversity Outbred founder strains. A) Schematic of study design to investigate insulin sensitivity and defensin protein expression in small intestines of inbred mouse strains. **B)** Matsuda Index in Diversity Outbred founder strains. **C)** Adiposity in Diversity Outbred founder strains. **D)** Correlations of all quantified defensin peptides (Defa) with

207 Matsuda Index across Diversity Outbred founder strains. **E)** Correlation of mean normalised alpha-
208 defensin 26 abundances with Matsuda Index in Diversity Outbred founder strains. **F)** Founder strain
209 contribution estimates for the DOz Matsuda Index QTL (top) and QTL LOD score (bottom) on
210 chromosome eight. **G)** Correlation of founder strain contribution estimates for the DOz Matsuda Index
211 QTL with mean normalised alpha-defensin 26 abundance. Dashed lines denote 95% confidence
212 intervals. Data are mean with biological replicates shown as individual data points. ** $P < 0.01$, * $P <$
213 0.05

214

215 *Alpha defensin 26 dietary supplementation improves insulin sensitivity in HFD-fed*
216 *C57BL/6J mice*

217 Genetic mapping in DOz mice and analysis of the founder strains revealed that alpha-
218 defensin 26 is a positive regulator of insulin sensitivity. To explore whether alpha-
219 defensin 26 could protect against diet-induced insulin resistance we undertook dietary
220 supplementation studies by synthesising the lumenal (to mimic post-processing
221 secretion) form of alpha-defensin 26 by 9-fluorenylmethyloxycarbonyl-solid-phase
222 peptide synthesis (Fmoc-SPPS), followed by folding (45). As a positive control, we
223 synthesised the lumenal form of human alpha-defensin 5 as this peptide has
224 previously been shown to improve glucoregulatory control in C57BL/6J mice (29).
225 Consistent with previous work (29), mice fed a western diet (WD) supplemented with
226 Fmoc-SPPS synthesised alpha defensin 5 (Defa5) had attenuated weight gain and
227 reduced adiposity but normal lean mass (Figure S2A-C) when compared to control
228 mice fed a control WD. Defa5 supplementation also improved insulin sensitivity,
229 evidenced by equivalent glucose tolerance but lower insulin levels relative to control
230 mice (Figure S2D, E). These results indicate that Fmoc-SPPS synthesised alpha-
231 defensin peptides behave comparably to previous peptides generated by traditional

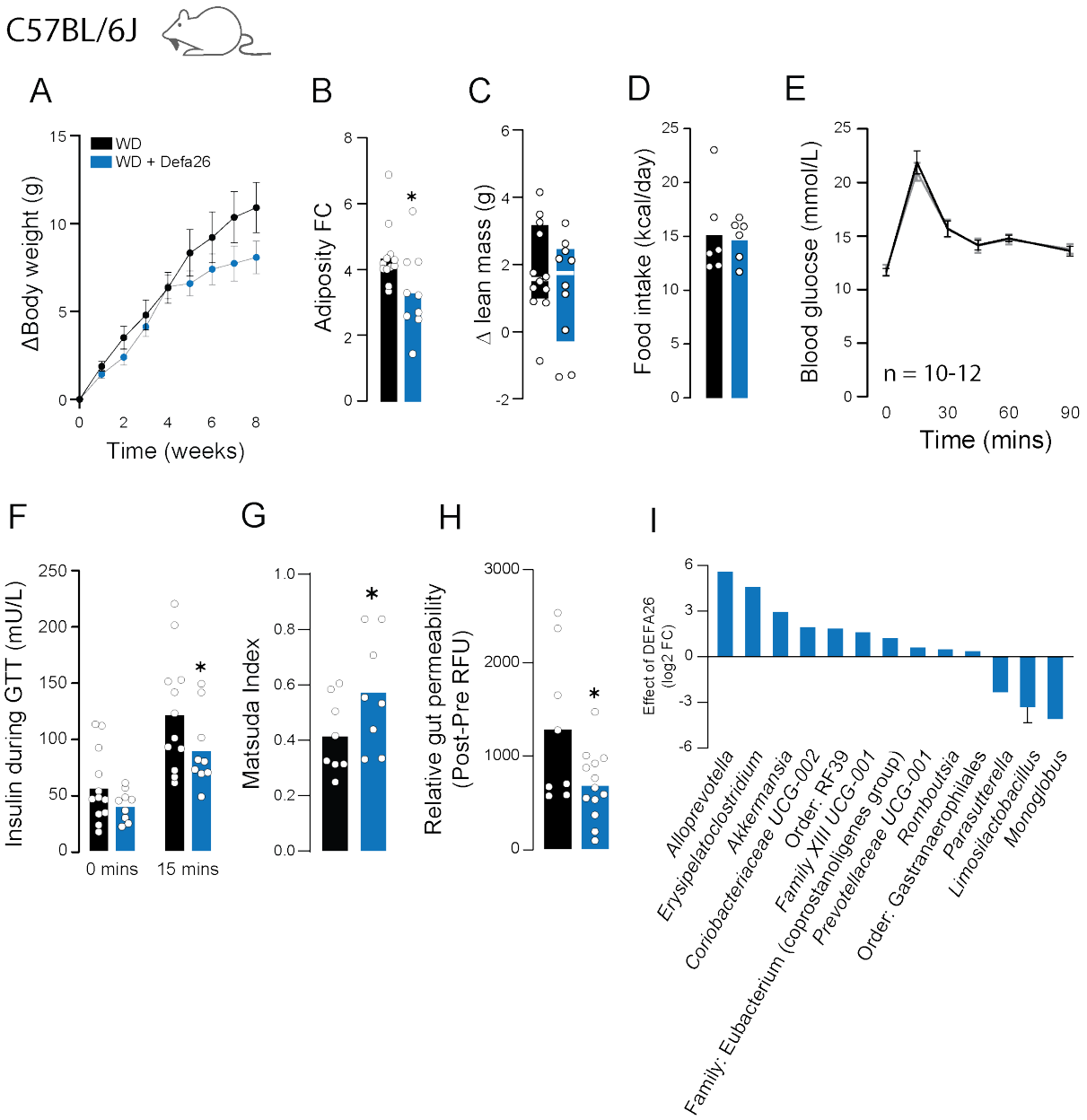
232 expression systems (26, 27, 29). Based on these results we proceeded with alpha-
233 defensin 26 supplementation (Defa26).

234

235 Male C57BL/6J mice were fed either a control WD or WD supplemented with alpha-
236 defensin 26 (WD + Defa26) for eight weeks. On average WD + Defa26 mice gained
237 less overall body mass and adipose tissue (Figure 4A, B), but had equivalent lean
238 mass relative to control WD fed mice (Figure 4C). This reduction in adipose tissue
239 does not appear to be the result of reduced food intake as this was comparable
240 between groups (Figure 4D). Although both groups exhibited near identical glucose
241 tolerance (Figure 4E), WD + Defa26 fed mice had lower circulating levels of insulin at
242 the 15 min timepoint of a GTT (Figure 4F) suggesting improved insulin sensitivity.
243 Consistent with this, the Matsuda Index was higher in WD + Defa26 fed mice relative
244 to WD fed controls.

245 In an attempt to profile potential mechanisms underpinning improved insulin sensitivity
246 in WD + Defa26 mice we measured gut permeability by measuring FITC fluorescence
247 in plasma, following oral gavage of FITC-dextran. Consistent with reduced gut
248 permeability, WD + Defa26 fed mice had lower fluorescence than WD fed controls.
249 Considering that genetic variance in the defensin locus associated with increased
250 abundance of certain metabolically beneficial microbes we carried out 16S rRNA
251 sequencing of caecal contents from WD and WD + Defa26 fed mice. In validation of
252 associations between defensin locus SNPs and microbial taxa, caecums from WD +
253 Defa26 fed mice were enriched for *A. muciniphila* and depleted of *Limosilactobaccilus*
254 *spp*. We also detected increased *Alloprevotella spp.* a microbe whose abundance has
255 previously been linked to defensin supplementation (29).

256 These results suggested that Defa26 supplementation could protect against WD
257 induced insulin resistance potentially via reduced adiposity and improved gut barrier
258 integrity. Furthermore, caecum microbial composition of WD + Defa26 fed mice
259 exhibits similar changes than the ones observed in mice harbouring a putative
260 protective SNP within the defensin locus.



261

262 **Figure 4 – Metabolic phenotyping of western-diet fed C57BL/6J mice supplemented with alpha-**
263 **defensin 26. A)** Change in body weight (g) of western diet (WD) or WD + alpha-defensin 26 (WD +
264 Defa26) fed C57BL/6J mice over eight weeks. **B)** Relative (fold-change) increase in adipose tissue

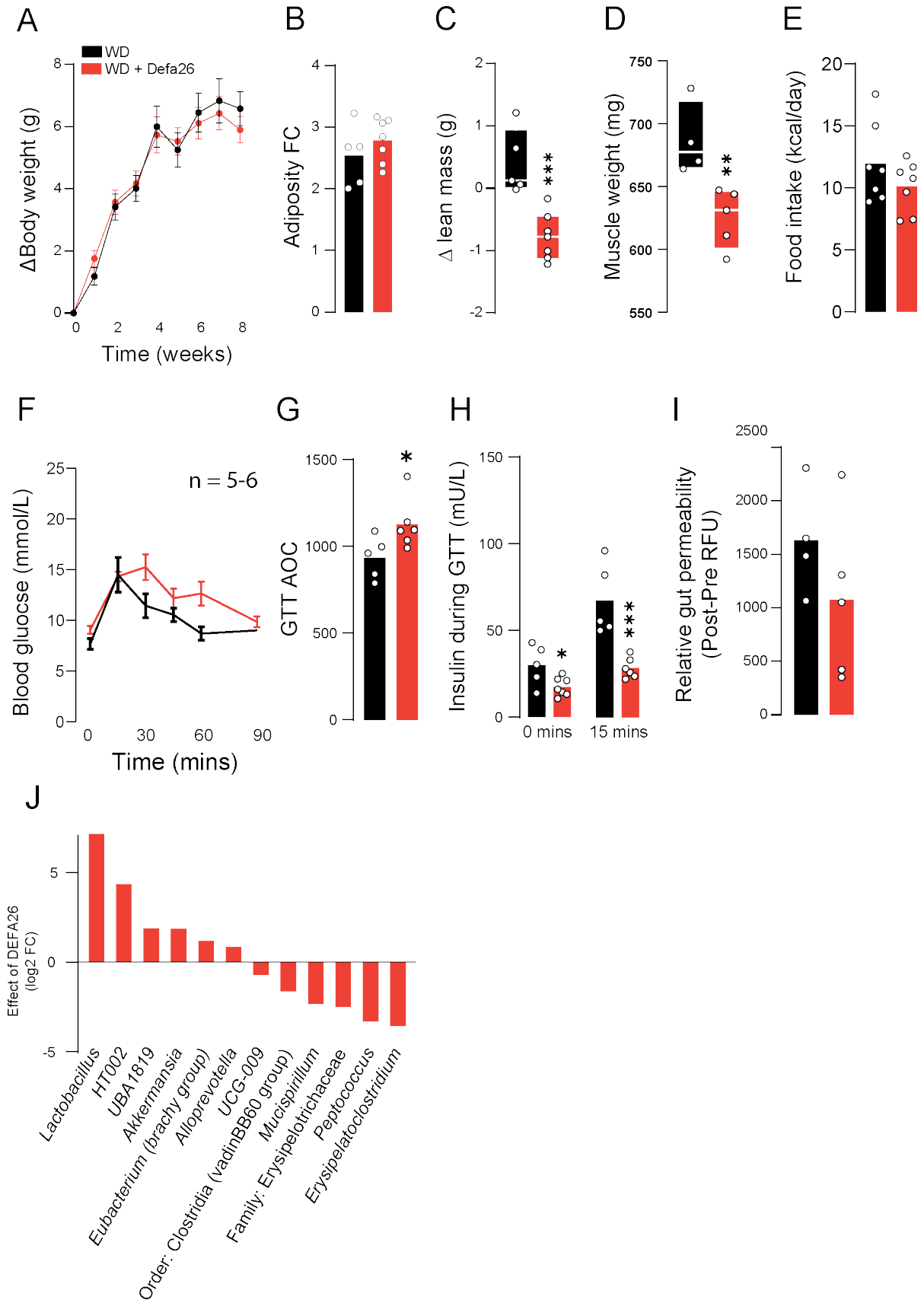
265 mass of WD and WD + Defa26 fed C57BL/6J mice over eight weeks. **C)** Change in lean mass (g) of
266 WD and WD + Defa26 fed C57BL/6J mice over eight weeks. **D)** Food intake (kcal/day) of WD and WD
267 + Defa26 fed C57BL/6J mice over eight weeks. **E)** Blood glucose concentrations during a glucose
268 tolerance test of C57BL/6J mice fed either a WD or WD + Defa26 for eight weeks. **F)** Blood insulin
269 concentrations during a glucose tolerance test of C57BL/6J mice fed either a WD or WD + Defa26 for
270 eight weeks. **G)** Matsuda Index of C57BL/6J mice fed either a WD or WD + Defa26 for eight weeks. **H)**
271 Relative gut permeability (post – pre FITC fluorescence) of C57BL/6J mice fed either a WD or WD +
272 Defa26 for eight weeks. **I)** Difference (log2 FC) in relative abundance of differential abundant microbes
273 between C57BL/6J mice fed either a WD or WD + Defa26 for eight weeks. Data are mean with biological
274 replicates shown as individual data points. For differentially abundant microbes, error bars represent
275 SD of difference between groups. * $P < 0.05$ denotes statistical significance from WD control.

276 *Alpha defensin 26 dietary supplementation induces hypoinsulinemia, glucose*
277 *intolerance and muscle wasting in WD-fed A/J mice*

278 In view of the responses observed in C57BL/6J mice, we next performed experiments
279 A/J mice. A/J mice are protected from diet-induced insulin resistance and express
280 relatively high levels of Defa26 and so we hypothesised that dietary supplementation
281 in this strain would have no effect on whole-body metabolism. Unlike in C57BL/6J
282 mice, A/J mice fed WD + Defa26 exhibited comparable weight gain to WD fed controls
283 (Figure 5A). Furthermore, adiposity increased to the same extent in both WD and WD
284 + Defa26 fed A/J mice (Figure 5B). However, A/J mice fed a WD + Defa26 exhibited
285 a striking (~1g) reduction in lean mass relative to WD fed controls (Figure 5C). This
286 decrease appears to be the result of muscle wasting, based on summed weights of
287 gastrocnemius, tibialis anterior and quadriceps muscles from WD and WD + Defa26
288 animals (Figure 5D). As with C57BL/6J mice, we did not observe a difference in food
289 intake between diets (Figure 5E).

290 To assess the effect of Defa26 on glucose homeostasis in A/J mice we performed
291 GTTs and observed relative fasting hyperglycaemia and glucose intolerance in WD +
292 Defa26 fed (Figure 5F,G). This appeared to be the result of hypoinsulinemia rather
293 than insulin resistance as WD + Defa26 fed A/J exhibited lower circulating insulin
294 levels both in fasting conditions and during a GTT. Unlike C57BL/6J mice, A/J mice
295 fed WD + Defa26 did not exhibit improvements in gut integrity over WD fed controls
296 (Figure 5I). Despite these differences in phenotypic response relative to C57BL/6J
297 mice, *A. muciniphila* and *Alloprevotella spp* were also enriched in the caecums of A/J
298 mice supplemented with Defa26, albeit to a lesser extent (Figure 5J).

A/J


A
WD
WD + Defa26
ΔBody weight (g)
Time (weeks)
B
Adiposity FC
C
Δ lean mass (g)
D
Muscle weight (mg)
E
Food intake (kcal/day)
F
n = 5-6
Blood glucose (mmol/L)
Time (mins)
G
GTT AOC
H
Insulin during GTT (mU/L)
0 mins
15 mins
I
Relative gut permeability (Post-Pre RFU)
J
Effect of DEFA26 (log2 FC)
Lactobacillus
HT002
UBA1819
Akkermansia
Eubacterium (brachy group)
Alloprevotella
UCG-009
Order: Clostridia (vadinBB60 group)
Mucispirillum
Family: Erysipelotrichaceae
Peptococcus
Erysipelatoclostridium

300 **Figure 5 – Metabolic phenotyping of western-diet fed A/J mice supplemented with alpha-**
301 **defensin 26. A)** Change in body weight (g) of western diet (WD) or WD + alpha-defensin 26 (WD +
302 Defa26) fed A/J mice over eight weeks. **B)** Relative (fold-change) increase in adipose tissue mass of
303 WD and WD + Defa26 fed A/J mice over eight weeks. **C)** Change in lean mass (g) of WD and WD +
304 Defa26 fed A/J mice over eight weeks. **D)** Combined mass of gastrocnemius, tibialis anterior and
305 quadriceps muscles from A/J mice after WD or WD + Defa26 feeding for 8 weeks. **E)** Food intake
306 (kcal/day) of WD and WD + Defa26 fed A/J mice. **F)** Blood glucose concentrations during a glucose
307 tolerance test of A/J mice fed either a WD or WD + Defa26 for eight weeks. **G)** Glucose tolerance test
308 'area-under-the-curve' for A/J mice fed either a WD or WD + Defa26 for eight weeks. **H)** Blood insulin
309 concentrations during a glucose tolerance test of A/J mice fed either a WD or WD + Defa26 for eight
310 weeks. **I)** Relative gut permeability (post – pre FITC fluorescence) of A/J mice fed either a WD or WD
311 + Defa26 for eight weeks. **J)** Difference (log2 FC) in relative abundance of differential abundant
312 microbes between A/J mice fed either a WD or WD + Defa26 for eight weeks. Data are mean with
313 biological replicates shown as individual data points. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ denotes
314 statistical significance from WD control.

315 *Disrupted microbial bile acid metabolism may explain the deleterious effects of alpha-*
316 *defensin 26 supplementation in A/J mice*

317 Despite our hypothesis that Defa26 supplementation would have little to no impact on
318 A/J mice, we in fact observed muscle wasting, glucose intolerance and
319 hypoinsulinemia in response to 8 weeks of dietary supplementation in this strain. This
320 contrasted sharply with the beneficial effects observed in C57BL/6J mice and
321 reinforces the importance of strain selection when testing potential therapeutics.

322 To begin understanding what might underpin these effects we looked to the
323 microbiome as this is the primary site of action for defensin peptides. Comparing the
324 differentially abundant microbes in C57BL/6J and A/J mice revealed relatively strain-
325 specific changes (Figure 6A,B). Only three microbes were changing concordantly in
326 both strains: *A. muciniphila* (Figure 6C), *Alloprevotella spp.* (Figure 6D), and

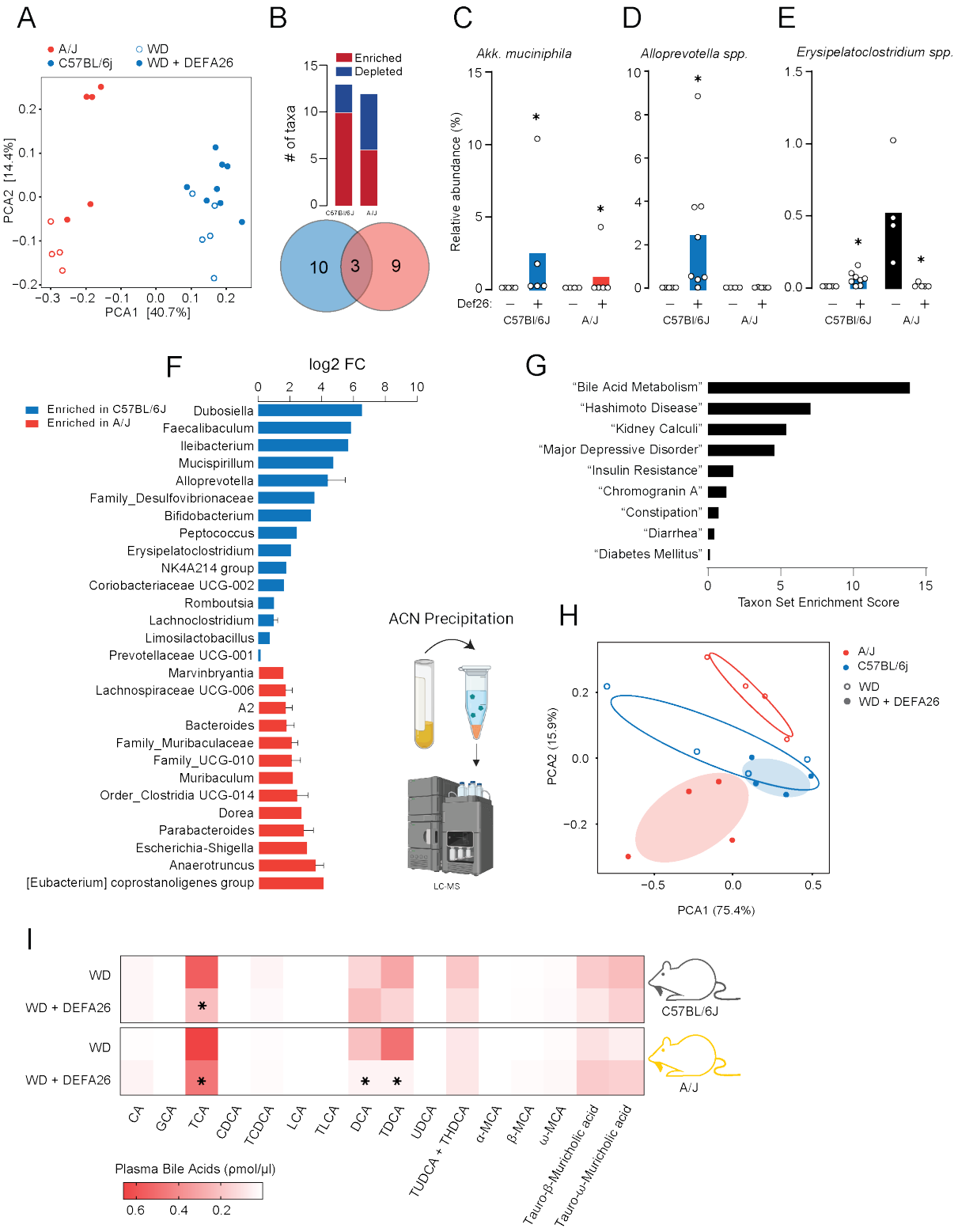
327 *Erysipelatoclostridium spp* (Figure 6E). Further reinforcing strain specificity,
328 *Erysipelatoclostridium spp* increased in C57BL/6J but was depleted by Defa26
329 treatment in A/J mice (Figure 6E).

330 To further investigate how microbiome-strain interactions could underpin the
331 differential effects of Defa26 supplementation we compared the microbiomes of
332 Defa26 treated C57BL/6 and A/J mice (Figure 6F). This revealed 28 differentially
333 abundant taxa, which were then analysed by taxon-set enrichment analysis (46).
334 Using the 'host-intrinsic' dataset revealed a striking enrichment for 'Bile Acid
335 Metabolism', as well as other relevant terms including 'Insulin Resistance' and
336 'Diabetes Mellitus' (Figure 6G). Based on this, and previous work linking microbially
337 derived bile acids to insulin secretion and lean mass (16, 47, 48) we set out to profile
338 circulating bile acids in Defa26 fed mice.

339 Using liquid chromatography-mass spectrometry (LC-MS) we measured the
340 abundance of 16 primary and secondary bile acids in plasma from a subset of
341 C57BL/6J and A/J mice fed either a WD or WD + Defa26 (S3A, B). Consistent with
342 strains-specific effects of Defa26 supplementation, principal component analysis could
343 separate A/J but not C57BL/6J bile acid profiles based on Defa26 supplementation
344 (Figure 6H). In A/J mice, Defa26 has a mild effect on taurocholic acid (TCA) levels, as
345 well as more striking reductions in deoxycholic acid (DCA) and taurodeoxycholic acid
346 (TDCA) (Figure 6I, S3B). Notably, both DCA and TDCA are derived from microbially
347 mediated deconjugation reactions and can activate the canonical bile acid receptor
348 FXR (farnesoid X receptor) (49). Previous work has shown that activation of FXR in
349 pancreatic beta-cells by bile acids is required for optimal glucose-stimulated insulin
350 secretion (48, 50) and bile-acid induced FXR signalling in the liver can modulate
351 muscle mass via FGF15/19 (17). Therefore, reductions in DCA and TDCA in A/J mice

352 due to disrupted microbial bile acid metabolism may explain the observed effects on

353 reduced insulin secretion and muscle mass.



354

355 **Figure 6 – Comparison of caecal microbiomes and circulating bile acids from C57BL/6J and A/J**
356 **following alpha-defensin 26 supplementation. A)** Visualisation of beta-diversity (ANCOM-BC) in WD
357 and WD + Defa26 fed C57BL/6J and A/J mice. **B)** Comparison of differentially abundant microbial taxa
358 in mice fed either a WD or a WD + Defa26 assessed by ANCOM-BC. **C)** *Akkermansia muciniphila*
359 relative abundance in C57BL/6J and A/J mice fed either a WD or a WD + Defa26. **D)** *Alloprevotella sp.*
360 relative abundance in C57BL/6J and A/J mice fed either a WD or a WD + Defa26. **E)**
361 *Erysipelatoclostridium spp.* relative abundance in C57BL/6J and A/J mice fed either a WD or a WD +
362 Defa26. **F)** Difference (log2 FC) in relative abundance of differential abundant microbes between
363 C57BL6/J and A/J mice fed WD + Defa26 for eight weeks. **G)** Enrichment scores for statistically
364 significant terms included in Taxon Set Enrichment Analysis. **H)** Principal component visualisation of
365 circulating bile acids in C57BL/6J and A/J mice fed either a WD or a WD + Defa26. **I)** Heatmap
366 visualisation of circulating bile acid concentrations in C57BL/6J and A/J mice fed either a WD or a WD
367 + Defa26. Data are mean with biological replicates shown as individual data points. * $P < 0.05$ denotes
368 statistical significance from WD control.

369

370 **Discussion**

371 Functional links between the gut microbiome and glucose homeostasis have
372 predominately been made via high fat/high sugar, low fibre 'western' diets, which bias
373 gut microbial composition towards an inflammatory obesogenic state (8, 51, 52). Here
374 we reveal an alternative gut-microbiome/metabolic health axis by performing genetic
375 mapping in a population of chow-fed DOz mice. We identified a striking insulin
376 sensitivity QTL within the defensin gene cluster, which associated with an enrichment
377 for metabolically beneficial microbes, and enhanced expression of the antimicrobial
378 peptide Defa26. We validated this observation by performing dietary supplementation
379 studies in two inbred mouse strains with differential endogenous Defa26 expression.
380 Our results revealed that Defa26 controls glucose homeostasis in an inverted U-
381 shaped relationship. At low levels, increasing concentrations of Defa26 improved gut

382 integrity and *A. muciniphila* abundance, whereas excess Defa26 disrupted microbial
383 bile acid metabolism, leading to insulin secretion defects and muscle wasting. This
384 illustrates the importance of considering genetic variation in the development of
385 metabolic therapeutics and places the microbiome downstream of host genetics in the
386 control of insulin sensitivity.

387

388 In our DOz colony, litters are separated at weaning to avoid confounding genetic
389 diversity with cage-effects. We took advantage of this to test whether the defensin
390 locus/insulin sensitivity association was microbiome mediated. We observed that
391 sharing a cage with an AB mouse conferred a mild beneficial effect on insulin
392 sensitivity and increased *A. muciniphila* abundance. This is consistent with previous
393 defensin supplementation studies (43, 53) and microbial transfer via coprophagy, and
394 suggests the mechanism linking the defensin locus to insulin sensitivity is microbiome
395 mediated. This has important implications beyond the present study as siblings of
396 different genotypes are commonly co-housed in traditional transgenic mouse
397 experiments. If there is an effect of genotype on gut microbial composition, any
398 subsequent effect on host-physiology may be masked by microbial transfer between
399 cage-mates. For example, a potential Defa26 knock-out mouse model co-housed with
400 wild-type control mice may appear phenotypically normal provided it maintains a
401 healthy microbiome via coprophagy.

402

403 The murine defensin cluster on chromosome 8 contains as many as 75 defensin
404 genes, 12 of which were located within 2 Mb of the insulin sensitivity Matsuda Index
405 QTL. To determine which of these were potentially mediating the association between

406 the defensin locus and insulin sensitivity we performed tandem mass spectrometry on
407 small intestine tissue in DOz founder strains. Taking this approach, only Defa26
408 associated with insulin sensitivity and QTL founder contributions suggesting that
409 genetic variants within the defensin locus promote both Defa26 expression and insulin
410 sensitivity. Defa26 was first identified in 2004 by a phylogenetic search of the *Mus*
411 *musculus* genome (54). Interestingly, the aforementioned study revealed that while
412 the mature defensin peptide sequence varies between isoforms, the signal peptide
413 and pro-peptide are highly conserved. This suggests that selection has optimised the
414 lumenal activity of defensins rather than their pre-secretion processing. Alignment of
415 the mature defensin peptide sequences identified in our study revealed that Defa26 is
416 unique relative to other defensins at the following residues V63G and K72T. Notably
417 the glycine at position 63 is near the N-terminus of the first beta sheet and may alter
418 interactions between the defensin 'barrel structure' and microbial membranes, leading
419 to selective antimicrobial activities and the observed effects on insulin sensitivity.

420

421 AB DOz mice and C57BL/6J mice supplemented with alpha-defensin 26 were both
422 more insulin sensitive than their relevant controls and their caecum was enriched for
423 the mucin-dwelling microbe *A. muciniphila*. As defensins are the most concentrated in
424 the mucin layer (22), it stands to reason *A. muciniphila* has evolved resistance against
425 defensin peptides that can be exploited by defensin administration for beneficial
426 metabolic effects by enabling *A. muciniphila* growth that may have been prevented by
427 other defensin sensitive microbes (11, 12, 43). Intriguingly, Zhang et al., (21) identified
428 several significant QTL for *A. muciniphila* in high-fat diet (HFD) fed DO mice. While
429 these QTL did not include the defensin locus, they did include the *Atf3* and *Tifa* loci
430 which are involved in Paneth cell differentiation. Differences between our study and

431 that of Zhang et al., likely reflect differences in diet and population genetic architecture,
432 but nevertheless, they both point towards an important role of Paneth cells and
433 defensins in *A. muciniphila* abundance.

434

435 In stark contrast with our hypothesis that A/J mice would not respond to Defa26
436 supplementation they exhibited hypoinsulinemia, muscle wasting and glucose
437 intolerance. Previous experiments in DO founder strains (16) and TSEA comparing
438 the gut microbiomes of Defa26 fed C57BL/6J and A/J mice indicated this could be due
439 to disrupted bile acid metabolism. Indeed, circulating levels of TCA, DCA and TDCA
440 were reduced by Defa26 supplementation in A/J but not C57BL/6J mice. This suggests
441 that Defa26 supplementation may disrupt microbes that facilitate the stepwise
442 deconjugation and 7α-dehydroxylation reactions ultimately required to convert cholic
443 acid (CA) into TDCA. (49, 55, 56). As agonists for the bile acid receptors FXR and
444 Tgr5, loss of DCA and TDCA may inhibit bile acid signalling in peripheral tissues (57),
445 such as beta-cells which require active FXR signalling for optimal insulin secretion (48,
446 50) and muscle tissue which exhibits atrophy upon Tgr5 ablation. This disruption may
447 not have occurred in C7BL6/J mice as they exhibit lower endogenous Defa26
448 expression which ultimately does not reach toxic levels following exogenous
449 supplementation.

450

451 There are two conclusions we draw from the present study. First, the gut microbiome
452 is a downstream effector of genetic variants which regulate insulin sensitivity. In our
453 data, microbial and metabolic differences between mice fed an identical diet can be
454 explained by genetic variance at a single locus. Historically, determining cause-and-

455 effect between microbes and insulin resistance has been challenging as both are
456 altered by diet. However, by anchoring upon genetics we can infer causality from
457 genotype to phenotype via the proteome and microbiome. Secondly, and perhaps
458 most importantly, the impact of individual biological differences on potential
459 therapeutic outcomes is significant, and must be considered as we move into the era
460 of preclinical precision medicine.

461 **Methods**

462 *Mouse breeding and phenotyping*

463 Male 'Diversity Outbred from Oz' (DOz) mice were bred and housed at the Charles
464 Perkins Centre, University of Sydney, NSW, Australia as previously described (39).
465 The DOz mice used in this study were outbred for 27 to 36 generations and comprised
466 a total of 670 male DOz mice across 9 separate cohorts. Genomic DNA was isolated
467 from each mouse and subjected to SNP genotyping (58), followed by genotyping
468 diagnostics and cleaning as described (59). Experiments were performed in
469 accordance with NHMRC guidelines and under approval of The University of Sydney
470 Animal Ethics Committee, approval numbers #1274 and #1988. To delineate genetic
471 from cage-effects, mice were randomised into cages of 3-5 at weaning. All mice were
472 maintained at 23°C on a 12-hour light/dark cycle (0600-1800) and given *ad libitum*
473 access to a standard laboratory chow diet containing 16% calories from fat, 61%
474 calories from carbohydrates, and 23 % calories from protein or a high-fat high-sugar
475 diet (western diet; WD) containing 45% calories from fat, 36% calories from
476 carbohydrate and 19% calories from protein (3.5%g cellulose, 4.5%g bran, 13%g
477 cornstarch, 21%g sucrose, 16.5%g casein, 3.4%g gelatine, 2.6%g safflower oil,
478 18.6%g lard, 1.2%g AIN-93 vitamin mix (MP Biomedicals), 4.95%g AIN-93 mineral mix
479 (MP Biomedicals), 0.36%g choline and 0.3%g L-cysteine). Fat and lean mass
480 measures were acquired via EchoMRI-900 (EchoMRI Corporation Pte Ltd, Singapore)
481 at 14 weeks of age. Glucose tolerance was determined by oral glucose tolerance test
482 (GTT) at 14-weeks of age by fasting mice for 6-hours (0700-1300 hrs) before oral
483 gavage of 20% glucose solution in water at 2 mg/kg lean mass. Blood glucose
484 concentrations were measured directly by handheld glucometer (Accu-Chek, Roche
485 Diabetes Care, NSW, Australia) from tail blood 0, 15, 30, 45, 60, 90 minutes after oral

486 gavage of glucose. Blood insulin levels at the 0- and 15-minute time points were
487 measured by mouse insulin ELISA Crystal Chem USA (Elk Grove Village, IL, USA)
488 according to manufacturer instructions. Blood glucose and insulin levels were
489 integrated into a surrogate measure of whole-body insulin sensitivity using the
490 Matsuda Index:

491 $$Matsuda\ Index = \frac{10{,}000}{\sqrt{(Glucose_0 \times Insulin_0) \times (Glucose_{\mathrm{GTT}\ mean} \times Insulin_{GTT\ mean})}}$$

492

493 *Genetic mapping analysis*

494 Genetic mapping of Matsuda Index was performed in R using the QTL2 package (60)
495 following square root transformation of raw values. The GIGA-MUGA single nucleotide
496 polymorphism array was used as genomic inputs for mapping (58), and a covariate
497 and kinship matrix to account for genetic relatedness amongst the DOz animals.
498 Significance thresholds were established by performing 1000 permutations and set at
499 $P < 0.05$.

500

501 *Caecal DNA isolation*

502 Genomic DNA was extracted from caecal contents of mice using the FastDNA Spin
503 Kit for Feces (MP Biomedicals) as per the manufacturers protocol. DNA concentration
504 was measured using the Qubit dsDNA BR assay kit (Invitrogen). Mock preparations
505 covering all steps of the procedure were conducted as contamination process controls.

506

507 *16S rRNA gene amplicon sequencing analysis*

508 16S rRNA gene amplicon sequencing was performed on all caecal DNA samples.
509 Barcoded amplicon libraries spanning the V4 hypervariable region of the 16S rRNA
510 gene were prepared (515F-806R primer set- 515F: GTGYCAGCMGCCGCGGTAA,
511 806R: GGACTACNVGGGTWTCTAAT) and sequenced using the Illumina MiSeq v2 2
512 x 250 bp platform at the Ramaciotti Centre for Genomics (UNSW, Sydney, Australia).
513 Raw sequence reads were processed using the DADA2 R package which involves
514 using error profiles to define Amplicon Sequence Variants (ASVs)(61). ASVs were
515 assigned to taxonomy using a pre-trained naïve Bayes classifier trained on the curated
516 16S rRNA gene SILVA (v138.1) reference database. Any ASV that was present in
517 fewer than 5% of samples or had less than 0.01% of total reads was filtered from the
518 final dataset prior to downstream analysis. Sequencing depth analyses and rarefaction
519 were performed with the *phyloseq* and *vegan* R package (62).

520 Analysis and graphical presentation of the resultant ASV data was performed in R
521 using the packages *phyloseq*, *vegan*, *microbiome* and *ggplot2*. Alpha diversity metrics
522 were calculated using Inverse Simpson's index. Beta diversity was assessed on
523 centred-log-ratio transformed ASV counts using Bray Curtis dissimilarity and UniFrac
524 distance and principal coordinate plots generated from the resultant dissimilarity
525 matrix. PERMANOVA (adonis) using the *vegan* R package was used to assess
526 variance in the distance matrices between groups. Differential abundance analysis
527 was performed using the *ANCOM-BC* R package(63).

528

529 *Intestinal proteomic sample preparation*

530 Division of the small intestine into thirds was achieved by folding the small intestine
531 into three equivalent lengths and taking a 1 cm section of tissue from the centre of

532 each third. These pieces tissue representing the foregut, midgut and hindgut of each
533 mouse were combined and snap frozen in liquid nitrogen. Frozen samples were then
534 boiled in 400 uL of SDC buffer (4% sodium deoxycholate, 100mM Tris-HCl pH 8.0) by
535 heating at 95 C for 10 minutes at 1000 rpm. Samples were then lysed by sonication
536 for 10 minutes (30 seconds on, 30 seconds off, 70% amplitude protocol). Samples
537 were then heated a second time at 95 ºC for 10 minutes at 1,000 rpm before being
538 clarified by centrifugation at 18,000 for 10 minutes at room temperature. Supernatant
539 was taken as lysate and protein concentration was determined by BCA assay, 10 µg
540 of protein was then prepared as previously described (30). Reduction/alkylation
541 (10mM TCEP, 40mM CAA) buffer was added to each sample before incubation for 20
542 minutes at 60 °C. Once cooled to room temperature, 0.4 ug trypsin and 0.4 ug LysC
543 was added to each sample and incubated overnight (18h) at 37 ºC with gentle
544 agitation. 30 µL water and 50 µL 1% TFA in ethyl acetate was added to stop digestion
545 and dissolve any precipitated SDC. Samples were prepared for mass spectrometry
546 analysis by StageTip clean up using SDB-RPS solid phase extraction material
547 (64).(64) Briefly, 2 layers of SDB-RPS material was packed into 200 µL tips and
548 washed by centrifugation at 1,000 x g for 2 minutes with 50 µL acetonitrile followed by
549 0.2% TFA in 30% methanol and then 0.2% TFA in water. 50 µL of samples were
550 loaded to StageTips by centrifugation at 1,000 g for 3 minutes. Stage tips were washed
551 with subsequent spins at 1,000 g for 3 minutes with 50 µL 1% TFA in ethyl acetate,
552 then 1% TFA in isopropanol, and 0.2% TFA in 5% ACN. Samples were eluted by
553 addition of 60µL 60% ACN with 5% NH_4OH_4. Samples were dried by vacuum
554 centrifugation and reconstituted in 30 µL 0.1% TFA in 2% ACN.

555

556 *Mass spectrometry analysis*

557 Peptides prepared as above (2 mg total), were directly injected using a Shimazu LC-
558 40 UHPLC onto a 5 cm x 2.1 mm C18 column analytical column (Agilent InfinityLab
559 Poroshell 120 EC-C18, 1.9 um particles) fitted with a 0.5 cm x 2.1 mm C18 guard
560 column (Agilent InfinityLab Poroshell 120 EC-C18, 1.9 um particles). Peptides were
561 resolved over a gradient from 3% - 36% acetonitrile over 10 min with a flow rate of 0.8
562 mL min^{-1}. Peptide ionization by electrospray occurred at 5.5 kV, with curtain gas 25,
563 gas 2 25 and gas 3 35. A 7600 Zeno TOF mass spectrometer (Sciex) with CID
564 fragmentation used for MS/MS acquisition. Spectra were obtained in a data-
565 independent acquisition using Zeno SWATH with 50 isolation width windows spanning
566 400 to 900 Th. Gas-phase fractionation of a pooled mixture of intestine peptides was
567 performed using 100 Th windows per run, to enable spectral library generation
568 covering the 400-900 Th range. Data files were analyzed using the quantitative DIA
569 proteomics search engine, DIA-NN (version 1.8.1) For spectral library generation, the
570 Uniprot mouse Swissprot database downloaded on the 1st of July 2022 was used.
571 Trypsin was set as the protease allowing for 1 missed cleavage and 1 variable
572 modification. Oxidation of methionine were set as a variable modification.
573 Carbamidomethylation of cystine was set as a fixed modification. Remove likely
574 interferences and match between runs were enabled. Neural network classifier was
575 set to double-pass mode. Protein inference was based on genes. Quantification
576 strategy was set to Robust LC (high accuracy). Cross-run normalization was set to
577 RT-dependent. Library profiling was set to full profiling.

578

579 *Peptide synthesis*

580 N,N-dimethylformamide (DMF) and dichloromethane (CH_2Cl_2) for peptide synthesis
581 were purchased from RCI Labscan and Merck. Gradient grade acetonitrile (CH_3CN)
582 for high-performance liquid chromatography was purchased from Sigma Aldrich and
583 ultrapure water (Type 1) was obtained from a Merck Milli-Q EQ 7000 water purification
584 system. Standard Fmoc-protected amino acids (Fmoc-Xaa-OH), coupling reagents
585 and resins were purchased from Mimotopes or Novabiochem. Fmoc-SPPS was
586 performed manually with these reagents and solvents in polypropylene Teflon-fritted
587 syringes purchased from Torviq and through automated synthesis on a SYRO I
588 peptide synthesizer (Biotage). Buffer salts for folding reactions were purchased from
589 Ajax, Sigma Aldrich, and Thermofisher and used as received. Glutathione reduced
590 and oxidised were purchased from Sigma Aldrich and Thermofisher, respectively. All
591 other reagents were purchased from AK Scientific or Merck and used as received.

592 Electrospray mass spectra (ESI-MS) were obtained using a Shimadzu 2020 UPLC-
593 MS with a Nexera X2 LC-30AD pump, Nexera X2 SPD-M30A UV/Vis diode array
594 detector, and a Shimadzu 2020 mass spectrometer using electrospray ionisation (ESI)
595 operating in positive mode. Separations were conducted using a Waters Acquity UPLC
596 BEH300 (1.7 μm, 2.1 x 50 mm C18 column) with a flow rate of 0.6 mL/min. Spectra
597 are recorded from 300-2000 Da.

598 Reverse-phase high performance liquid chromatography (RP-HPLC) was carried out
599 on a Waters 2535 Quaternary Gradient system, fitted with a Waters 2489 UV/Vis
600 Detector (monitoring at 214 and 280 nm) and a Waters Fraction Collector III. Linear
601 peptides were purified by preparative RP-HPLC using an Xbridge C18 column (5 μm,
602 19 × 150 mm) at a flow rate of 12 mL/min. A mobile phase of Milli-Q water (Solvent A)
603 and HPLC-grade CH_3CN (Solvent B) was employed over a linear gradient with 0.1
604 vol% TFA (trifluoroacetic acid) as an additive.

605 Folded peptides were purified by semi-preparative RP-HPLC using a Xbridge C18
606 column (5 µm, 10 × 250 mm) at a flow rate of 4 mL/min. A mobile phase of Milli-Q
607 water (Solvent A) and HPLC-grade CH_3CN (Solvent B) was employed over a linear
608 gradient with 0.1 vol% TFA as an additive.

609 Analytical RP-HPLC was performed on a Waters Alliance e2695 HPLC system
610 equipped with a 2998 PDA detector (λ = 210–400 nm). Separations were performed
611 on a Waters Sunfire® C18 (5 µm, 2.1 × 150 mm) column at 40 ºC with a flow rate of
612 0.5 mL/min. All separations were performed using a mobile phase of 0.1% TFA in
613 water (Solvent A) and 0.1% TFA in CH_3CN (Solvent B) using linear gradients.

614 Peptides were synthesized on a 50 µmol scale. 2-Chlorotrityl chloride (2-CTC) resin
615 was treated with Fmoc-Xaa-OH (1.2 eq) and *i*-Pr_2NEt (4.8 eq) in DCM (4 mL). The C-
616 terminal amino acid of each sequence was used for this loading step, ie. Fmoc-Leu-
617 OH was used for the loading of Defa26 and Fmoc-Arg(Pbf)-OH was used for Defa5.
618 Fmoc loading was determined by measuring the piperidine fulvene adduct from Fmoc
619 deprotection. The loading of the resins were: 0.51 mmol/g for Leu on CTC (Defa26)
620 and 0.50 mmol/g for Arg on CTC (DEFA5).

621

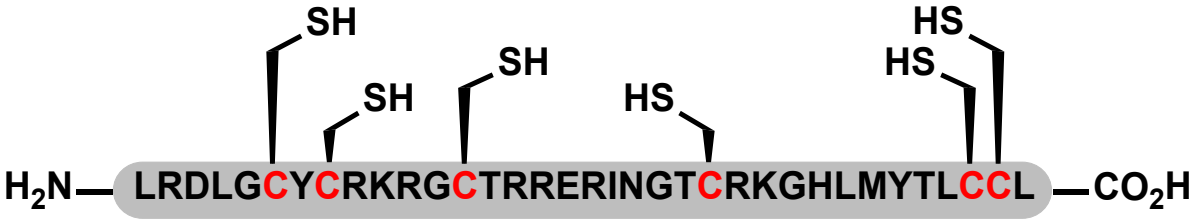
622 <u>General procedure A: Automated Fmoc-Solid-Phase Peptide Synthesis (SPPS) –</u>
623 <u>SYRO I automatic peptide synthesizer (Biotage).</u>

624 50 µmol of the amino acid loaded resin was treated with a solution of piperidine (40
625 vol%, 0.8 mL) in DMF for 3 min, drained, before repeat treatment with piperidine (20
626 vol%, 0.8 mL) in DMF for 10 min. The resin was then drained and washed with DMF
627 (4 x 1.2 mL) before addition of a solution of Fmoc-Xaa-OH (200 µmol, 4 eq.) and
628 Oxyma (4.4 eq.) in DMF (400 µL), followed by a solution of *N,N'*-

629 diisopropylcarbodiimide (4 eq.) in DMF (400 µL). The resin was then agitated at 75 °C
630 for 15 min or 50 °C for 30 min as specified [coupling of Fmoc-His(Trt)-OH and Fmoc-
631 Cys(Trt)-OH were reacted at 50 °C for 30 min in all instances]. The resin was then
632 drained via vacuum and one repeat treatment of the coupling conditions was
633 conducted. The resin was then washed with DMF (4 x 1.2 mL) before being treated
634 with a solution of 5 vol% Ac_2O and 10 vol% *i*-Pr_2NEt in DMF (1.6 mL) and agitated for
635 5 min to cap unreacted N-terminal amines on the growing peptide. The resin was then
636 drained and washed with DMF (4 x 1.6 mL). Iterative cycles of this process were
637 repeated until complete peptide elongation was achieved after which the resin was
638 washed with DMF (4 x 5 mL) and CH_2Cl_2 (5 x 5 mL). HCl counterion exchanges were
639 performed by dissolving the folded peptide in 0.1 M HCl and lyophilising on a freeze
640 drier. This HCl treatment and lyophilisation was repeated 6 times.

641 Synthesis of Defa26

642

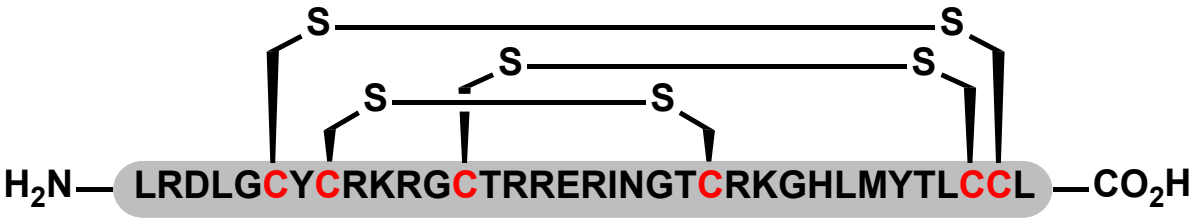
643 

644 Scheme 1. Linear sequence of Defa26.

645 The above linear sequence was synthesised according to General Procedure A on 2-
646 CTC resin which was loaded with Fmoc-Leu-OH. The peptide was then cleaved from
647 resin by treatment with 85:5:5:5 v/v/v/v TFA/triisopropylsilane/H_2O/ethanedithiol for 2
648 h at rt. The cleaved solution was collected, dried to ~1 mL under N_2 flow and the
649 peptide product was precipitated using diethyl ether (2 x 40 mL) and collected *via*

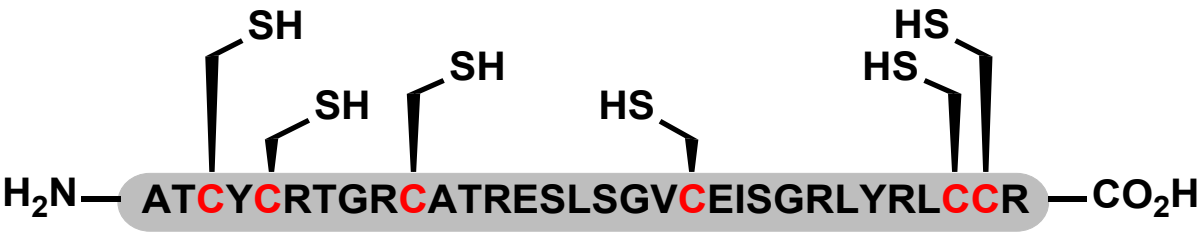
650 centrifugation. The crude linear peptide was then purified by preparative RP-HPLC (0
651 vol% CH_3CN + 0.1 vol% TFA for 10 min, then 0-50 vol% CH_3CN + 0.1 vol% TFA over
652 50 min) and lyophilised affording the linear Defa26 as a white solid (10.14 mg, 4%).

653 Linear Defa26 (5.5 mg, 1 eq) was first dissolved in 220 µL of rapid dilution buffer
654 containing TRIS (50 mM), NaCl (150 mM), guanidine.HCl (6 M), and tris(2-
655 carboxyethyl)phosphine (2 mM). This solution was then added gradually to a buffer
656 containing $NaHCO_3$ (200 mM), urea (2 M), GSH (1 mM), and GSSG (0.2 mM) in MilliQ
657 water to make up a 1 mg/mL peptide solution. The folding reaction was left for 16 h
658 without stirring. The folding progress was monitored through LC-MS, and a loss of 6
659 Da and a simultaneous retention time shift indicated completion of folding. The folded
660 peptide was then purified by semi-preparative RP-HPLC (0 vol% CH_3CN + 0.1% TFA
661 for 20 min, then 0-50 vol% CH_3CN + 0.1 vol% TFA over 50 min), affording the folded
662 Defa26 as a white solid after lyophilization (1.07 mg, 19% isolated yield). Prior to
663 biological assays, the peptide was converted to the HCl salt through a HCl counterion
664 exchange.

665 

666 Figure S2. Crude semi-preparative scale trace of folded Defa26. The first 20 minutes
667 of the 0 vol% B + 0.1 % TFA wash is omitted for clarity. Arrow indicates the collected
668 folded peptide.

669 <u>Synthesis of DEFA5</u>

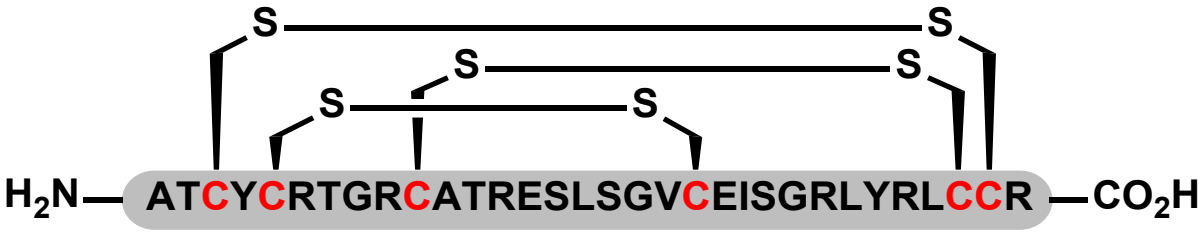
670 

671 Scheme 3. Linear sequence of DEFA5.

672 The above linear sequence was synthesised according to General Procedure A on 2-
673 CTC resin which was loaded with Fmoc-Arg(Pbf)-OH. The peptide was then cleaved
674 from resin by treatment with 85:5:5:5 v/v/v/v TFA/triisopropylsilane/H_2O/ethanedithiol
675 for 2 h at rt. The cleaved solution was collected, dried to ~1 mL under N_2 flow and the
676 peptide product was precipitated using diethyl ether (2 x 40 mL) and collected *via*
677 centrifugation. The crude linear peptide was then purified by preparative RP-HPLC (0
678 vol% CH_3CN + 0.1 vol% TFA for 10 min, then 0-50 vol% CH_3CN + 0.1 vol% TFA over
679 50 min), affording the linear DEFA5 as a white solid after lyophilisation (3.19 mg, 3%).

680 Linear DEFA5 (2.51 mg, 1 eq) was first dissolved in 100 µL of rapid dilution buffer
681 containing TRIS (50 mM), NaCl (150 mM), guanidine.HCl (6 M), and tris(2-
682 carboxyethyl)phosphine (2 mM). This solution was then added gradually to a buffer
683 containing NH_4OAc (330 mM), guanidine.HCl (500 mM), GSH (1 mM), and GSSG (0.2
684 mM) in MilliQ water to make up a 1 mg/mL peptide solution. The folding reaction was
685 then left for 40 h without stirring. The folding progress was determined through LC-
686 MS, and a loss of 6 Da and a simultaneous retention time shift indicated folding. The
687 folded peptide was then purified by semi-preparative RP-HPLC (0 vol% CH_3CN + 0.1%
688 TFA for 20 min, then 0-50 vol% CH_3CN + 0.1 vol% TFA over 50 min), affording the
689 folded DEFA5 as a white solid after lyophilisation (0.62 mg, 24% isolated yield). Prior
690 to biological assays, the peptide was converted to the HCl salt through a HCl
691 counterion exchange.

692



693

694 Scheme 4. Folded sequence of DEFA5.

695

696 *Defensin feeding experiments*

697 Prior to allocation into experimental groups, C57BL/6J and A/J mice underwent
698 baseline metabolic phenotyping was described above. Mice of each strain were then
699 fed a WD or a WD containing the luminal forms of either murine alpha-defensin 26
700 (Defa26) or human alpha-defensin 5 (DEFA5) for 8 weeks. Synthetic peptides were
701 mixed into mouse diet by hand. Even distribution of peptides in food was monitored
702 by the addition of blue food dye which was used as a proxy for the distribution of
703 peptides throughout each batch. After eight weeks mice underwent a second bout of
704 metabolic phenotyping and assessment of gut permeability via FITC-dextran oral
705 gavage as previously described (65). Briefly mice were fasted for 4 hours (0900-1300)
706 before a baseline blood sample (50 uL) was taken from a tail incision. Mice were then
707 gavaged with 150 µl of 80 mg/ml FITC dextran (4kDa). After 4 hours, a second blood
708 sample was taken. Both samples (baseline and post-gavage were then centrifuged at
709 5,000 rpm for 10 minutes. Resulting plasma was then diluted 1:10 in PBS and
710 fluorescence was measured at 530 nm with excitation at 485 nm. Data was then
711 expressed as relative fluorescence units.

712

713 *Bile acid extraction*

714 50 µL of plasma thawed on ice was added to 150 µL of ice-cold acetonitrile containing
715 5 pmoles of d4-cholic acid internal standard. Samples were vortexed for 30 s at
716 maximum speed then centrifuged at 15,000 x *g* for 10 min at 4°C to pellet insoluble
717 debris. 170 µL of supernatant was transferred to fused-insert HPLC vials, then vacuum
718 centrifuged to dryness in an Eppendorf Concentrator Plus. Samples were
719 reconstituted in 50 µL of 80:20 water:acetonitrile. All solvents were MS grade.

720

721 *Bile acid quantification*

722 Separation of bile acids was performed using a Nexera LC-40 UHPLC (Shimadzu,
723 Rydalmere, NSW, Australia) using a 2.1x 50 mm, 2.7 µm CORTECS C18 column
724 (Waters, Rydalmere, NSW, Australia) with a 7-minute binary gradient of 0.1% formic
725 acid in water (A) and acetonitrile (B) at a flow rate of 0.9 mL/min. Initial gradient
726 conditions of 83:17 A/B rose to 30% B at 1.2 min using curve setting 9. From 1.2 to
727 3.0 minutes, the proportion of B increased to 38% using curve -5, then rose to 100%
728 at 4.3 min using curve setting 5. The column was flushed at 100% B for 1.9 min before
729 returning to initial conditions over 0.1 min and being held for 0.7 min. Column
730 temperature was 50°C and injection volume was 0.5µL.

731 MS data were acquired on a ZenoTOF 7600 (Sciex, Mulgrave, VIC, Australia)
732 quadrupole-time-of-flight tandem MS operating with electrospray ionisation in negative
733 polarity. Intact bile acid precursor ions were detected using a TOF MS experiment with
734 mass range 200-600 Da and accumulation time 0.3 s. Source parameters were: Spray
735 voltage: -4500 V, Temperature: 650°C, Ion source gas 1: 70psi, Ion source gas 2:
736 80psi, Curtain gas: 40psi. Declustering potential was set to -80 V, collision energy was

737 -10 V and CAD gas was set to 10 (arbitrary units). MS calibration was maintained by
738 Calibrant Delivery System auto-calibration at intervals of approximately 1 hour.

739 Raw data were acquired in a single batch with acquisition order randomised. Six
740 replicates of a sample pool were distributed through the batch to assess intra-batch
741 imprecision. Six replicate injections from a single vial were acquired to determine
742 instrument repeatability. Data analysis was performed with the Analytics module of
743 SCIEX OS (version 3.1.6). Chromatographic peaks were extracted with a width of 0.02
744 Da and integrated using the AutoPeak algorithm. Identification of bile acids was based
745 on both accurate precursor m/z and retention time matched to commercial standards
746 for all quantified bile acid species (Table 1). Relative molar amounts for bile acids were
747 calculated by comparing raw peak areas relative to the internal standard. Average
748 mass accuracy was >1 ppm, with a range of +/- 2ppm across the run. %CVs were
749 calculated as peak area ratios relative to the internal standard.

750 **Table 1.** Bile acid identification details and coefficients of variation

Bile Acid	Mass (Da) [M-H]-	Retention Time (min)	Peak Width (min)	Inter-Batch (%CV)	Intra-Batch (%CV)
d4 CA	411.3116	2.36	0.15		
CA	407.2803	2.36	0.11	1.17	2.15
DCA	391.2854	3.93	0.19	0.99	6.09
CDCA	391.2854	3.74	0.16	3.63	2.21
UDCA	391.2854	2.45	0.13	5.47	4.9
αMCA	407.2803	1.95	0.08	5.52	12.23

ßMCA	407.2803	2.01	0.09	1.81	2.45
ωMCA	407.2803	1.91 and 2.01	0.08	1.93	2.53
GCA	464.3018	1.94	0.08	1.66	3.25
LCA	375.2905	4.44	0.05	13.49	25.22
TCA	514.2844	1.71	0.10	2.17	5.32
TDCA	498.2895	2.1	0.15	1.99	6.75
TCDCA	498.2895	2.01	0.13	2	5.05
THDCA	498.2895	1.69	0.17	2.29	4.56
TUDCA	498.2895	1.69	0.17	2.29	4.56
TLCA	482.2946	2.88	0.29	1.93	10.3
TßMCA	514.2844	1.53	0.09	2.59	5.47
TωMCA	514.2844	1.5	0.07	3.65	5.89

Data analysis

All data analysis and visualisation were performed in either the R programming environment (66) or GraphPad Prism (GraphPad Software, San Diego, California USA). For protein correlation analysis the Matsuda Index was calculated using glucose tolerance data before being log2 transformed. To correct for multiple testing, p-values were adjusted using the q-value method in the R package *qvalue* (67).(67) Chi-square tests for distribution differences within the data and two/one-way ANOVA tests for group differences were performed in GraphPad Prism.

760 **Acknowledgements**

761 We'd like to thank the Sydney Mass Spectrometry facility and Large Animal Services
762 in the Charles Perkins Centre at the University of Sydney for mass spectrometry and
763 mouse housing support.

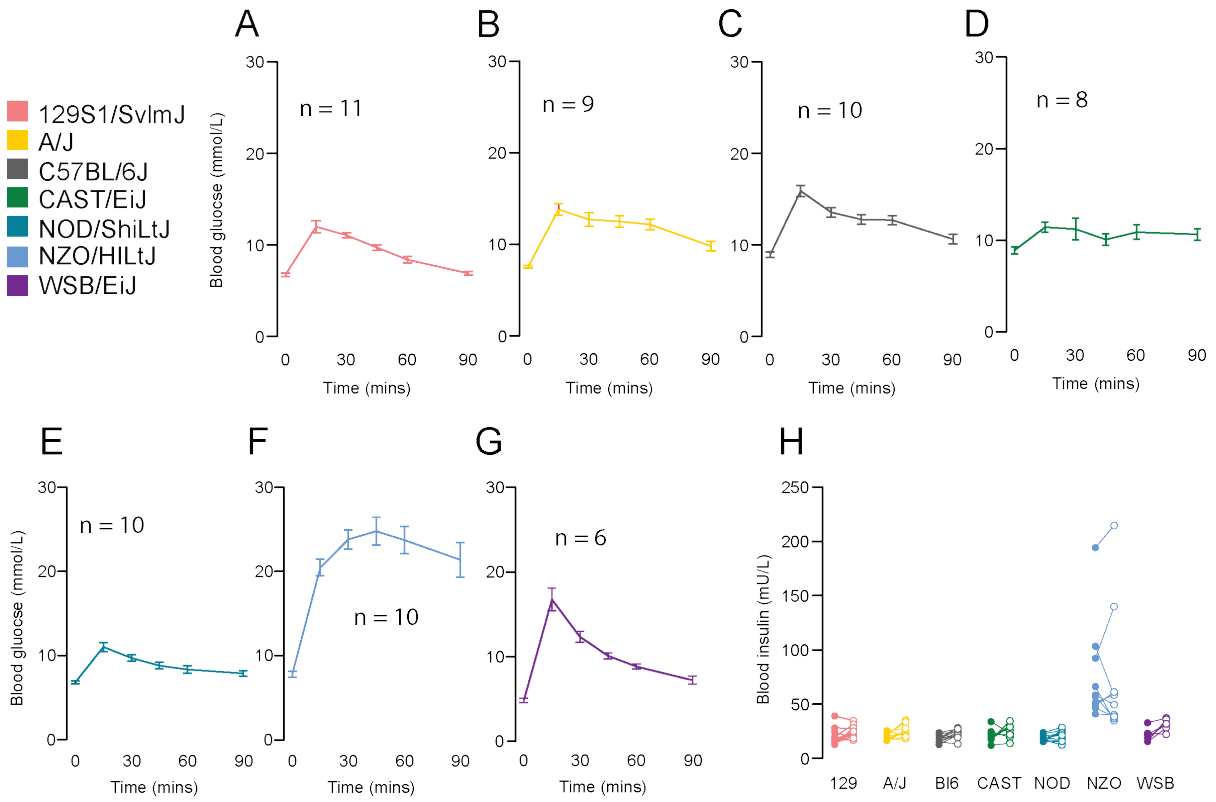
764

765 **Funding**

766 This work was supported by the Australian Research Council (Laureate Fellowship) to
767 DEJ and by Diabetes Australia (General Grant) to SWCM.

768

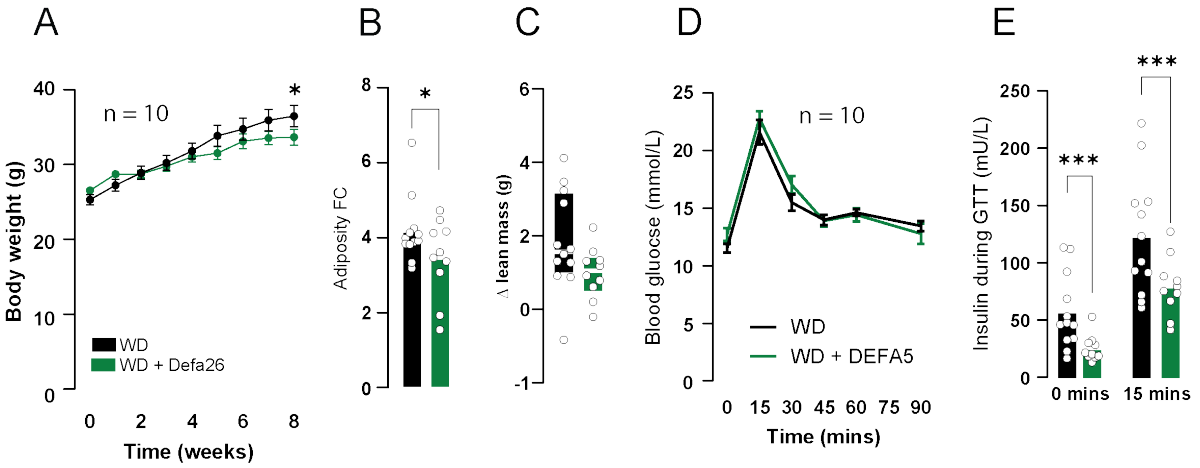
769 **Supplementary Figures**



770

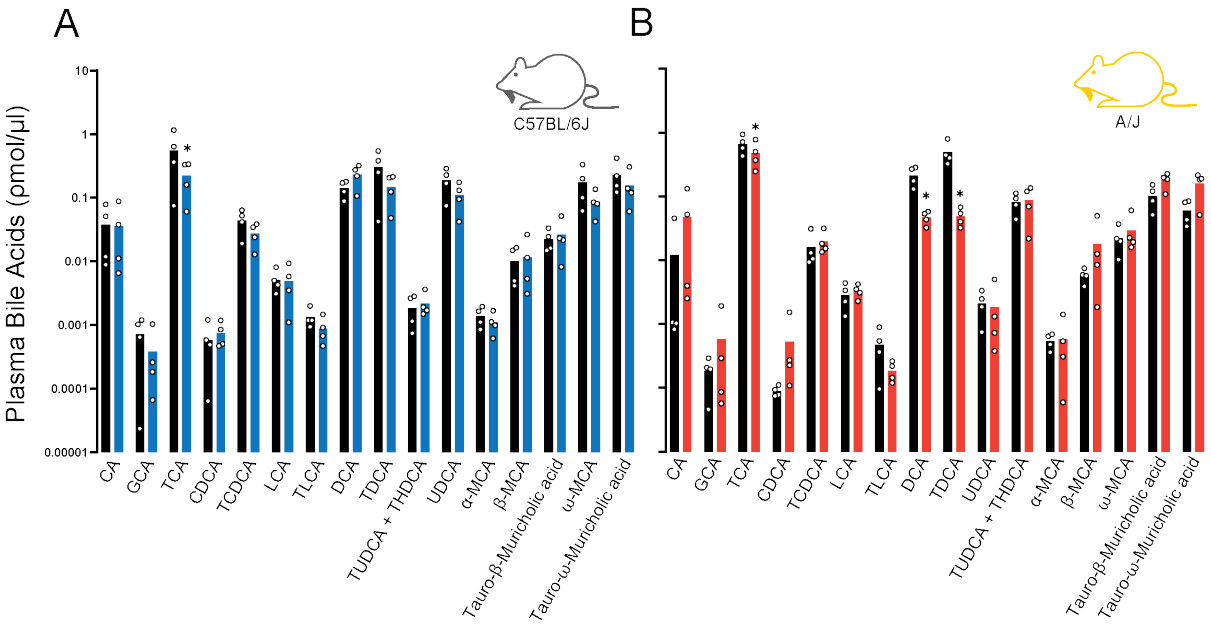
771 **Supplementary Figure 1 – Glucose tolerance and blood insulin concentrations**
772 **in Diversity Outbred founder strains.** Blood glucose concentrations during a

773 glucose tolerance test in **A)** 129S1/SvlmJ, **B)** A/J, **C)** C57BL/6J, **D)** CAST/EiJ, **E)**
774 NOD/ShiLtJ, **F)** NZO/HILtJ, **G)** WSB/EiJ. **H)** Insulin concentration in Diversity Outbred
775 founder strains during a glucose tolerance test. Data are mean with biological
776 replicates are shown as individual data points or noted in figure. **** P < 0.0001, *** P
777 < 0.001, ** P < 0.01, * P < 0.05



778

779 **Supplementary Figure 2 – Effect of alpha-defensin 5 supplementation on insulin sensitivity and**
780 **body composition in C57BL/6J mice. A)** Body weight of mice fed either WD or WD+DEFA5. **B)** Fold-
781 change in adiposity in mice fed either WD or WD+DEFA5. **C)** Change in lean mass in mice fed either
782 WD or WD+DEFA5. **D)** Blood glucose concentrations in mice fed either WD or WD+DEFA5 during a
783 glucose tolerance test. **E)** Insulin concentrations in mice fed either WD or WD+DEFA5 during a glucose
784 tolerance test. Data are mean with biological replicates are shown as individual data points or in figure.
785 *** P < 0.001, * P < 0.05



Supplementary Figure 3 – Effect of alpha-defensin 5 supplementation on insulin sensitivity and body composition in C57BL/6J mice. A) Plasma bile acid concentrations from C57BL/6J mice fed either a WD or WD + Defa26 for eight weeks. **B)** Plasma bile acid concentrations from A/J mice fed either a WD or WD + Defa26 for eight weeks. Data are mean with biological replicates are shown as individual data points or in figure. * $P < 0.05$ denotes a significant difference from WD fed mice.

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