eLife

Reviewed Preprint
v1 • December 22, 2025
Not revised

For correspondence:
adam.dobson@glasgow.ac.uk

Competing interests: No competing interests declared

Funding: See page 29

Reviewing editor: Scott F Leiser, University of Michigan, United States



# Microbiota impact *Drosophila* ageing via *Acetobacter*, *Tachykinin*, and *TkR99D*

**Diana Marcu[1], David R Sannino[1], Anthony J Dornan[1,2], Rita Ibrahim[1], Atharv Kapoor[1], Miriam Wood[1], Adam J Dobson[1]**

[1]School of Molecular Biosciences, College of Medical Veterinary and Life Sciences, University of Glasgow, Glasgow, United Kingdom • [2]Cellular Analysis Facility - Shared Research Facilities, College of Medical Veterinary and Life Sciences, University of Glasgow, Glasgow, United Kingdom

## eLife Assessment

This **important** study demonstrates that in Drosophila melanogaster, tachykinin (Tk) expression is regulated by the microbiota. The authors present **convincing** evidence that axenic flies raised with no microbiota are longer-lived than conventionally reared animals, and that Tk expression and Tk receptors in the nervous system are required for this effect. They further test individual bacterial strains for their role in these effects and connect the effect to loss of lipid stores and suggest that FOXO may be involved in the phenotype, results that are of interest to the fields of environmental perception, host microbiome interactions, and geroscience.

[Editors' note: this paper was reviewed by *Review Commons*.]

https://doi.org/10.7554/eLife.109547.1.sa4

## Abstract

Gut microbiota exert an evolutionarily conserved influence on ageing, from invertebrates to humans. How do microbes that are physically confined to the gut lumen affect the systemic physiological process of ageing? In female *Drosophila*, we show that microbiota increase expression of the peptide hormone *Tachykinin (Tk)*, which corresponds to reduced lifespan. *Tk* is required for microbiota to shorten lifespan, with knockdown rendering flies constitutively long-lived even in the presence of an intact microbiota. This lifespan extension does not come with canonical costs to fecundity or feeding, but impacts on triacylglyceride (TAG) storage suggest adaptive functions in metabolic homeostasis. In flies with defined (gnotobiotic) microbiotas, we show that we can model *Tk*-dependent effects of microbiota on lifespan and TAG by monoassociation with *Acetobacter pomorum*. These effects require *Tk* in the midgut, and the cognate TK receptor *TkR99D* in neurons, implicating a microbiota-gut-neuron relay. This relay also appears to compromise gut barrier function in aged flies, indicating roles in healthspan as well as lifespan. However, the effect of *TkR99D* is independent of its reported role in insulin signalling and adipokinetic hormone signalling which, respectively, are canonical regulators of lifespan and TAG metabolism, suggesting a non-canonical role for *TkR99D* elsewhere in the nervous system. Altogether our results implicate a microbiota-gut-neuron axis in ageing, via a specific bacterium modulating activity of a specific and evolutionarily-conserved hormone.

## Introduction

Gut microbiota influence lifespan and ageing across species. This effect appears to be evolutionarily conserved from the invertebrate models *Caenorhabditis elegans* and *Drosophila melanogaster* (Brummel et al., 2004; Cabreiro et al., 2013; Houthoofd et al., 2002; Matthews et al., 2020; Obata et al., 2018; Pryor et al., 2019; Sannino et al., 2018), through to vertebrates including fish and rodents (Gordon et al., 1966; Smith et al., 2017; Snyder et al., 1990; Tazume et al., 1991; Valenzano et al., 2015). Microbiota are also thought to play a role

in human ageing (Johansen et al., 2023; OToole and Jeffery, 2015), supported by mendelian randomisation analysis indicating a putatively causal role (Liu et al., 2023) (but see also (Gagnon et al., 2023)). Gut microbiota are physically confined to the gut lumen, yet they influence ageing at an organismal level. By what mechanisms do microbiota modulate these systemic processes?

Evolutionary theories of ageing lead us to expect that mechanisms of ageing should have adaptive functions early in life, i.e. processes under positive selection in early life have deleterious pleiotropic effects in later life, which manifest as ageing (Williams, 1957). Microbiota conform to this expectation, with their impacts on ageing appearing to trade off against widespread benefits to host health early in life. For example, microbiota commonly promote host development time, even with the same species of bacteria demonstrated to benefit host development across different phyla of animals (Schwarzer et al., 2016; Storelli et al., 2011; A. Wong et al., 2014). This motivates research to characterise how to decouple beneficial effects of microbiota early in life from deleterious effects in late life.

The capacity of microbiota to impact systemic processes, from a physically confined niche in the intestinal lumen, implicates endocrine signalling between organs. A role mediated by endocrine signalling is also consistent with the pleiotropic effects of microbiota, since hormones potently impact multiple traits. The relationship between microbiota and endocrine signalling may be bidirectional, with the microbiota altering how the gut - the biggest endocrine organ in animals - communicates lumen conditions to distal tissues, while distal tissues may direct how the gut manages the microbiota. Signalling in either direction has the potential to modulate ageing, with hormones serving as a key relay.

We chose to investigate these processes in the fruitfly *Drosophila melanogaster*. Flies can be reared axenic (i.e. without exogenous bacteria), and have a simple microbiota dominated by two families of bacteria (*Acetobacteraceae* and *Lactobacillaceae*). These bacteria can be cultured easily, and reintroduced to axenic fly eggs to generate gnotobiotic animals, enabling mapping of host phenotype to bacterial species. Fly genetics also enable tissue- and cell-type-specific genetic manipulations, e.g. expressing RNAi in specific locations, ideal for studying tissue-tissue signalling. We capitalised on these strengths to identify how defined bacteria modulate hormone signalling, and how ageing phenotypes are modulated by tissue-specific lesions in this signalling.

# Results

## A role for *Tk* in lifespan regulation by microbiota

First, we looked for evidence that microbiota modulate fly endocrine signalling. Hormone activity both to and from the gut could be relevant to impact of microbiota; but since the gut is the site of physical contact with gut microbiota, and also the largest endocrine organ, in which numbers of enteroendocrine cells (EEs) can vary plastically in adults, we reasoned that EE counts could serve as a proxy for microbial impacts of overall endocrine signalling capacity, even though other endocrine tissues could also be affected. When we counted absolute numbers of EEs in whole guts (Figure 1A), we found more cells in guts of conventionally-reared (CR) flies than in axenic flies, suggesting that microbiota enhance capacity for endocrine signalling. Previous studies have shown that microbiota reduce the percentage of EEs (Broderick et al., 2014; Liu et al., 2022), which we suggest may have been driven by an even greater microbial promotion of other epithelial cell populations, reducing proportion of EEs despite increased total numbers of EEs.

EEs express 14 peptide hormones (Hung et al., 2020). We asked whether microbiota promote expression of any of these specifically, by reanalysing public transcriptome data (Bost et al., 2018) (Figure 1B). We were able to quantify expression of 13 genes, and we analysed the fourteenth (*Burs*) by RT-qPCR (Figure S1). Four hormone genes were differentially expressed. Of these four, Tachykinin (*Tk*) - which encodes orthologues of the mammalian neuropeptides Substance P and Neurokinins A/B - appeared a particularly interesting candidate, because published data indicated that *Tk* knockdown flies phenocopy the longer lifespan and metabolic phenotypes observed in axenic flies (Dobson et al., 2015; Rewitz et al., 2024; Sannino et al., 2018; Song et al., 2014; A. C.-N. Wong et al., 2014). Since the differential expression analysis

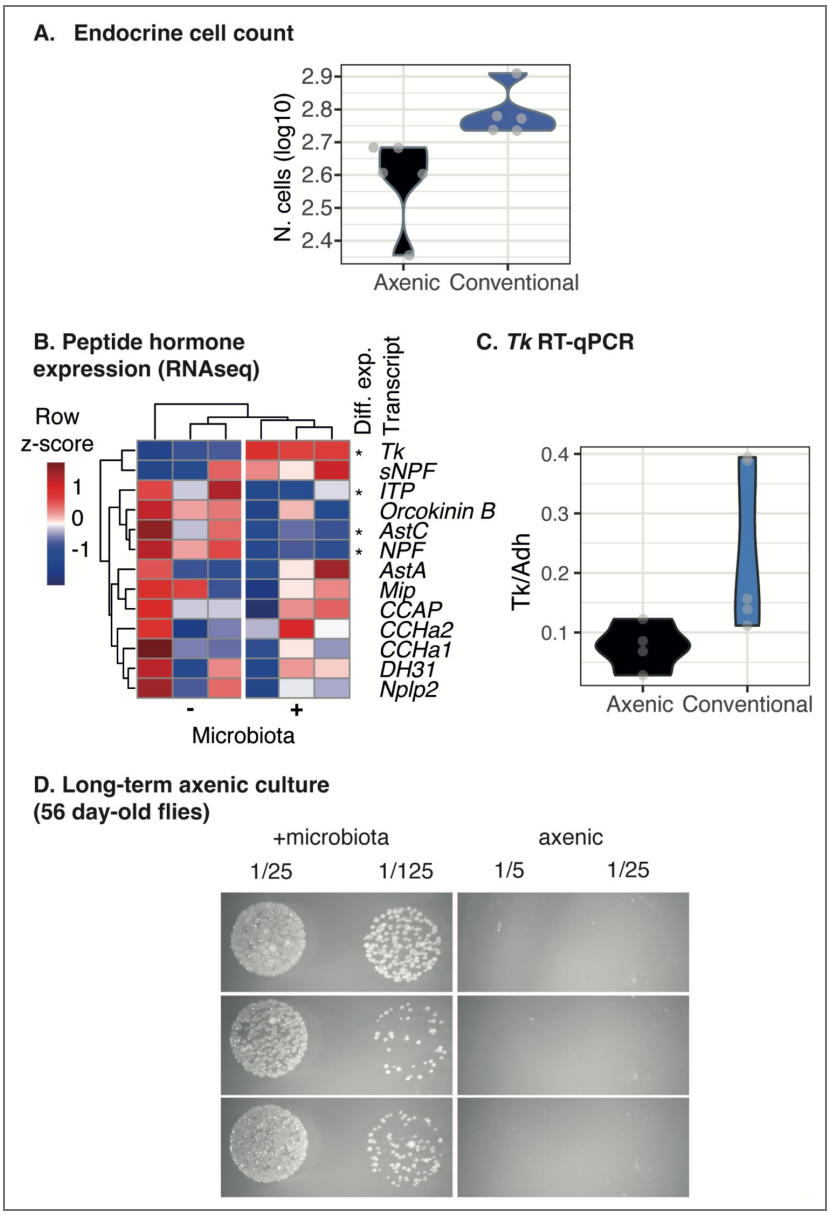



**Figure 1. Basis for study: evidence that *Tk* responds to microbiota.**

**A.** Microbiota increase total number of enteroendocrine cells (EEs) in fly midgut. Number of GFP+ cells were scored in 5-day old *UAS-mCD8::GFP; Voila-Gal4* females. Wilcoxon test, W=25, P=0.008. **B.** Microbiota alter expression of peptide hormones in the *Drosophila* intestine. Transcriptome data from (Bost et al., 2018) were reanalysed. Expression of neuropeptide hormone gene transcripts are plotted, dendrograms show hierarchical clustering on Euclidian distance. Differentially expressed transcripts (DESeq2, FDR<0.1) are marked with an asterisk to right. Presence of microbiota increased expression of *Tachykinin* (top row). Software did not quantify *Burs* expression, so complementary RT-qPCR data were collected, showing no impact of microbiota (Figure S1). **C.** Confirmation that microbiota promote *Tk* expression. Expression was quantified by RT-qPCR (using cDNA generated from midgut to complement RNA-seq data). Wilcoxon test W=1, p=0.04. **D.** Confirmation of lifelong axenic culture without antibiotics. Photographs show bacterial growth (or lack of) in homogenates of pools of 3 females, reared to day 56 of adulthood either with bacteria or axenic, serial dilutions indicated at top.

was performed on public data, we validated the impact of microbes on *Tk* expression in our lab stock (*Dahomey*) by RT-qPCR (Figure 1C). This correlation between microbial promotion of *Tk* expression and shortening of lifespan, coupled to phenocopying between axenic and *Tk* knockdown flies, led us to predict that *Tk* knockdowns would be constitutively long-lived and their lifespan should no longer respond to microbiota.

We tested our prediction by assessing impact of *Tk* knockdown in flies reared with/without a microbiota. We reared flies under axenic conditions from embryo until death, to avoid potential off-target effects on the fly of antibiotic feeding (Chatzispyrou et al., 2015; Moullan et al., 2015; Ronayne et al., 2023), after confirming that we could maintain sterile culture conditions throughout the lifespan (Figure 1D). To establish the genetic role of *Tk*, we first used a ubiquitous knockdown, to avoid in the first instance the possibility that targeting one specific tissue may lead to compensatory expression in another. We drove RNAi expression using an inducible ubiquitous driver (*Daughterless-GeneSwitch* a.k.a. *DaGS)*, which we activated in 3-day-old adult females by feeding a chemical inducer, RU486 (henceforth, RU), thereby avoiding any possible confounding impacts of knockdown during development. In all experiments, we used female flies and a fully-factorial design.

Microbiota shortened lifespan relative to axenic controls (Figure 2A). However, $Tk^{RNAi}$ blocked this effect, rendering CR flies as long-lived as axenics. Cox Proportional Hazards analysis (CoxPH) revealed a significant $Tk^{RNAi}$:microbiota interaction (p=0.05). To visualise specifically how the experimental conditions differed from one another, we used estimated marginal means (EMMs) to calculate *post hoc* differences between conditions, and to plot *post hoc* metrics of differences in survival (multiplied by −1 such that higher values corresponded to extended lifespan). This analysis revealed a significant shortening of lifespan by microbiota in CR flies relative to axenics, but $Tk^{RNAi}$ blocked this effect (Figure 2B), i.e. $Tk^{RNAi}$ extended lifespan in conventional flies, but not in axenic flies. To confirm the efficacy of *Tk* knockdown in conventional flies with an independent tool, we expressed a distinct RNAi in conventional flies, which also extended lifespan (Figure S3); and an independent recent study (Ahrentløv et al., 2025) also corroborates our findings. CR lifespan was not extended by RU in the absence of the *UAS-*$Tk^{RNAi}$ transgene (Figure S4), indicating that the *Tk:*microbiota interaction was attributable to knockdown, not off-target effects of RU feeding; and bacterial load was not altered by $Tk^{RNAi}$ induction, suggesting that the lifespan difference is not due to altered density of microbiota (Figure S5). Together, these results revealed that gut-microbial modulation of lifespan requires *Tk*.

## Evidence for pleiotropic roles of *Tk* between lifespan and metabolism, but not canonical tradeoffs with ageing

Tachykinins are highly pleiotropic peptides (Nässel, 2025), and interventions that extend lifespan are commonly associated with pleiotropic trade-offs early in life. For example, lifespan is expected to anticorrelate reproduction in early life (Partridge et al., 2005), while feeding behaviour, naturally selected to optimise growth and reproduction, is expected to be deleterious for ageing (Flatt, 2009; Good and Tatar, 2001; Grandison et al., 2009; Mair et al., 2005). We therefore hypothesised that $Tk^{RNAi}$ might block beneficial effects of microbiota on reproduction or feeding in early life. We measured steady-state feeding behaviour (proboscis extension) and a point measure of fecundity (egg laying over 24h), after one week of $Tk^{RNAi}$ induction in both axenic and CR flies. As expected, microbiota did indeed increase feeding and egg laying in CR flies relative to axenics (Figure 2C-D), but $Tk^{RNAi}$ did not reduce either fecundity or feeding. Thus, longevity in CR $Tk^{RNAi}$ flies appears not to be associated with canonical costs.

We looked for alternative pleiotropic traits. Microbiota have evolutionarily-conserved effects on host metabolism, with increased lipid storage observed in axenic hosts (Dobson et al., 2015; Turnbaugh et al., 2006; Vijay-Kumar et al., 2010). Since *Tk* is also implicated in mobilising fly triglyceride (TAG) stores (Song et al., 2014), we anticipated a potential tradeoff with lifespan. Without $Tk^{RNAi}$, microbiota reduced TAG storage as expected, but $Tk^{RNAi}$ blocked this effect in two

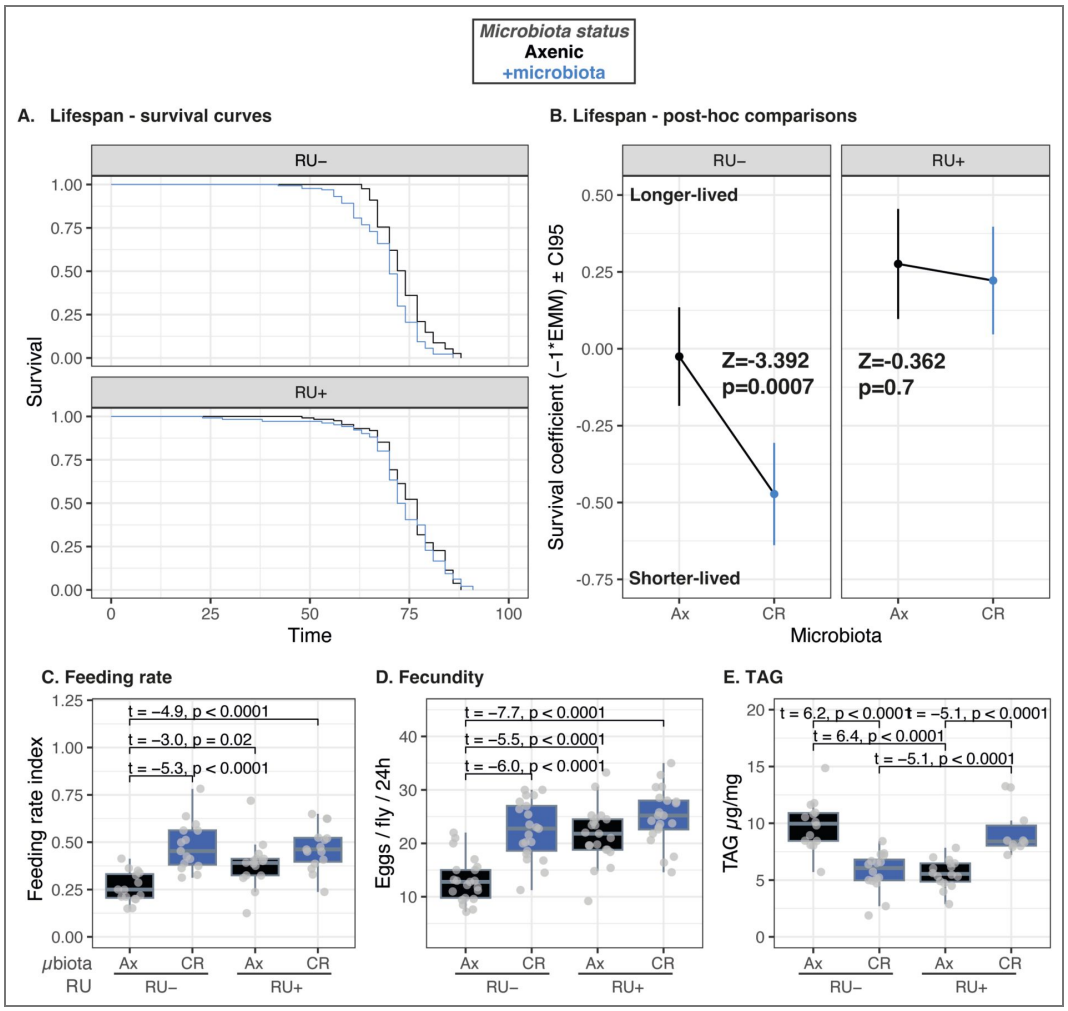



**Figure 2. Ubiquitous *Tk* is required for microbiota to shorten lifespan and reduce TAG storage.**

**A.** Kaplan-Meier plots showing survival of axenic and conventionally-reared (CR) *DaGS;UAS-Tk*$^{RNAi}$ flies, faceted by absence/presence (top/bottom) of transgene activation by RU486 (RU). Cox Proportional Hazards analysis revealed an RU:microbiota interaction (p=0.05). Axenic RU- n=135, axenic RU+ n=136, CR RU- n=135, CR RU+ n=135. **B.** Post-hoc analysis of survival data shown in A. Facets show estimated marginal means of Cox Proportional Hazards model (EMMs±95% confidence intervals (CI95)), multiplied −1*n to make higher values correspond to longer lifespan. Pairwise tests (text on facets) revealed impact of microbiota only in absence of RU. **C-D.** *Tk*$^{RNAi}$ (RU+) does not block effect of microbiota on feeding rate (C) or fecundity (D). Feeding quantified as average proboscis extension rate per vial (n=5 mated females/vial, 10 days old after 7 days feeding on RU/vehicle.). Fecundity quantified as n. eggs laid per fly over 24h (n=5 females/vial). **E.** *Tk*$^{RNAi}$ (RU+) blocks reduction of TAG by microbiota. Points give TAG µg mg$^{-1}$ in pools of five flies. The result was replicated with an independent RNAi construct (Figure S3). Panels A-E generated with RNAi construct V330743. Panels A-B, n=135-136 flies per condition. Panels C-E, Statistics on brackets are from *post-hoc* EMM analyses of linear models (C, logit-transformed data), when significant differences were detected.

replicate experiments with distinct $Tk^{RNAi}$ constructs (Figure 2E, Figure S6). These results suggested a pleiotropic role for *Tk* in managing metabolic responses to microbiota in early life, with deleterious impacts for lifespan.

## *Acetobacter pomorum*, but not *Levilactobacillus brevis*, interacts with *Tk* to determine lifespan and TAG

Can the interaction between microbiota and *Tk* be attributed to particular gut bacteria? The fly microbiota, while relatively simple, still contains numerous taxa, which vary among strains and environments, but tend to contain families *Acetobacteraceae and Lactobacillaceae* (Brown et al., 2023; Chandler et al., 2011; Staubach et al., 2012; Wang and Staubach, 2018; Wong et al., 2013). We characterised the fly microbiota in our lab background by 16S rRNA amplicon sequencing, and identified amplicon sequencing variants (ASVs). Most ASVs were assigned to the family *Acetobacteraceae* (Figure 3a), and *Lactobacillaceae* were also present (among other families). *Acetobacter pomorum* and *Levilactobacillus brevis* are two well-studied species that can be considered representatives of their respective genera, on the basis of genome content (Newell et al., 2014) and phenotypic impacts (Newell and Douglas, 2014). We asked whether either *A. pomorum* or *L. brevis* alone recapitulated impacts of a CR microbiota on lifespan and TAG, and whether they interacted equivalently with $Tk^{RNAi}$.

We generated gnotobiotic flies, monoassociated with either *A. pomorum* (DmCS004) or *L. brevis* (DmCS003), along with axenic controls, and tested whether lifespan impacts were blocked by ubiquitous $Tk^{RNAi}$ (Figure 3B-C). Without $Tk^{RNAi}$, axenic flies were longest-lived, and both bacteria shortened lifespan. flies associated with *A. pomorum* (henceforth *Ap*-flies) were shortest-lived, and flies associated with *L. brevis* (*Lb*-flies) were intermediate. However, $Tk^{RNAi}$ induction rendered flies constitutively long-lived, independent of microbiota association and equivalent to axenic controls. Thus, $Tk^{RNAi}$ blocked variation in lifespan caused by synthetic variation in the microbiota, and this effect was strongest in *Ap*-flies.

We quantified *Tk* expression by RT-qPCR among axenic flies, *Lb*-flies and *Ap*-flies, and asked whether change in expression upon knockdown (*DaGS, UAS-*$Tk^{RNAi}$) correlated lifespan outcome (Figure S7). *Tk* expression was lowest in axenics, highest in *Ap*-flies, and intermediate in *Lb*-flies (Figure S7A), but RU feeding arrested *Tk* expression across all three microbiota conditions - confirming universally efficient knockdown - and this expression level was equivalent to control axenic flies (Figure S7A). Plotting *Tk* expression against statistical coefficients (EMMs) of the corresponding survival data, revealed a correlation across conditions between *Tk* expression values and lifespan (Figure S7B). Thus, microbial modulation of lifespan appears to correspond to organismal expression of *Tk*.

We then asked whether specific bacteria and *Tk* interactively modulated TAG levels in youth (Figure 3E). We found reduced TAG in *Ap*-flies relative to axenic flies, and this effect was reversed by $Tk^{RNAi}$, consistent with the pleiotropic tradeoff we had observed in CR flies. However no interaction was apparent between *L. brevis* and $Tk^{RNAi}$. Thus, *A. pomorum* was sufficient to modulate TAG in interaction with *Tk*, but *L. brevis* was not. We also note that in both this experiment and the equivalents in CR flies (Figure 2E, Figure S6), feeding RU to axenic flies reduced TAG. Since $Tk^{RNAi}$ does not reduce *Tk* expression in axenics (Figure S7), we interpret this as a possible off-target effect of RU, however such an effect cannot account for the lack of difference between axenics without RU and *Ap*-flies/*Lb*-flies with RU.

Altogether, these data demonstrated that *A. pomorum* is sufficient to fully recapitulate the impacts of a complete microbiota on both lifespan and TAG, and interacts equivalently with ubiquitous $Tk^{RNAi}$. By comparison the intermediate effects in *Lb*-flies argues against *Lactobacillaceae* playing a leading role in the *Tk*-dependent regulation of lifespan and TAG.

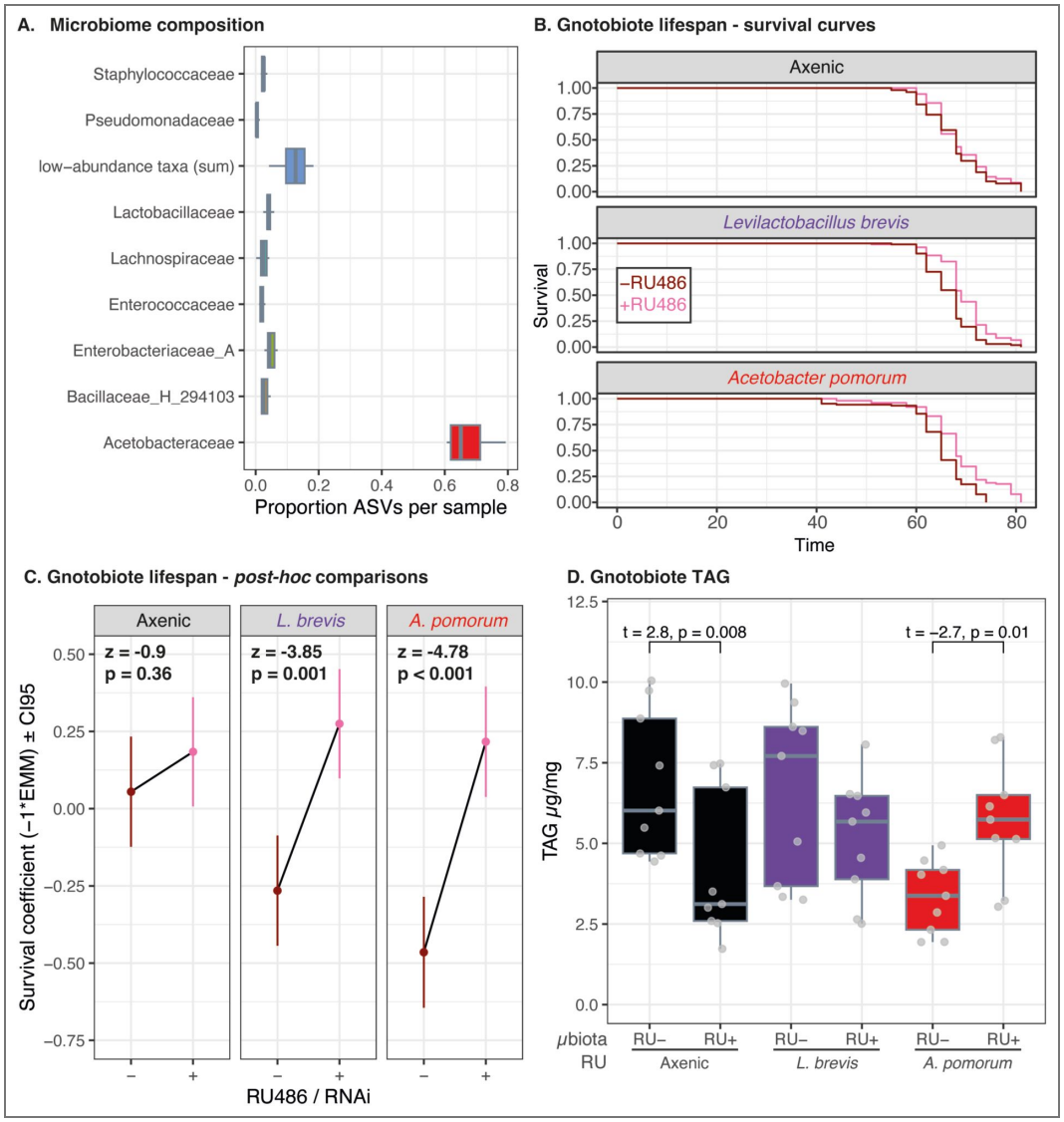



**Figure 3. Phenotypic impact of a complete microbiota and interaction with ubiquitous *Tk*$^{RNAi}$ is recapitulated fully by *Acetobacter pomorum*, but only partially by *Levilactobacillus brevis*.**

**A.** 16s rRNA amplicon sequencing reveals microbiota dominated by family *Acetobacteraceae* in the *Dahomey* fly background. Plot shows proportion ASVs per sample assigned to given families. "low abundance taxa" denotes a bin of all ASVs not assigned to one of the given families. ~170k reads per sample, n=10 samples. **B.** Kaplan-Meier plots showing survival of *DaGS;UAS-Tk*$^{RNAi}$ flies (RNAi construct V330743), cultured either under axenic or gnotobiotic conditions. Gnotobiotic flies were cultured in monoassociation with either *Acetobacter pomorum* (*Ap*-flies) or *Levilactobacillus brevis* (*Lb*-flies). Plots are faceted by microbial condition. Cox Proportional Hazards analysis revealed an RU:microbiota interaction (p=0.02). Ap RU- n=105, Ap RU+ n=105, Ax RU- n=105, Ax RU+ n=104, Lb RU- n=105, Lb RU+ n=105. **C.** Post-hoc analysis of survival curves shown in B. Plots show a survival coefficient, calculated from estimated marginal means of Cox Proportional Hazards model (EMMs±95% confidence intervals (CI95)), multiplied −1*n such that higher values correspond to longer lifespan. Pairwise tests (text on facets) revealed effect of *Tk*$^{RNAi}$ induction (+RU) was largest in *Ap*-flies, intermediate in *Lb*-flies, and no effect was observed in axenics. D. *A. pomorum,* but not *L. brevis,* recapitulates effects of a complete microbiota on TAG. Points give TAG µg mg$^{-1}$ in pools of five flies (n=9). Significant interaction of microbiota and *TkRNAi* (RU) was detected (ANOVA F=6.96, df=2,48, p=0.002). Statistics on brackets are from *post-hoc* EMM analyses of linear models, when significant differences were detected.

## *A. pomorum* modulates *Tk* expression in midgut but not CNS

Our initial experiments employed ubiquitous $Tk^{RNAi}$. From which specific tissue does *Tk* knockdown mediate effects of the microbiota? Plotting steady-state *Tk* expression from the FlyAtlas2 database (Leader et al., 2018) (i.e. from CR flies), revealed that *Tk* is enriched in neuronal and midgut tissues (Figure 4A). We therefore characterised metrics of Tachykinin activity from these two tissues in axenic flies, *Lb*-flies and *Ap*-flies. We examined *Tk* expression by RT-qPCR of RNA from heads and on dissected midguts. *A. pomorum* increased *Tk* expression in midgut, but not in heads; while *L. brevis* did not impact *Tk* expression in either tissue (Figure 4B). We used reporters to confirm intestinal modulation of *Tk* expression. *Acetobacter* are thought to promote Tk signalling epigenetically, by promoting histone lysine acetylation in *Tk*+ cells (Jugder et al., 2021). We confirmed that lysine acetylation was labile in *Tk*+ EEs by feeding axenic flies with a histone deacetylase inhibitor, trichostatin-A, and quantifying staining in *Tk*+ cells in both anterior and posterior regions of the midgut (Figure 4C-D). We then examined effects of microbiota, finding increased acetyl-lysine in *Tk*+ cells of *Ap*-flies, but not *Lb*-flies (Figure 4D). We asked whether this increase corresponded to elevated *Tk* expression, and indeed *Tk* reporter expression (*Tk-T2A-Gal4>UAS*-GFP) was elevated in the anterior midgut, but not posterior midgut (Figure 4E). Together, these results confirm previous observations that *A. pomorum* increases lysine acetylation in *Tk*+ EEs, suggesting possible epigenetic regulation or priming of *Tk* regulation, but correspondence between lysine acetylation and *Tk* reporter expression appears specific to certain regions of the midgut.

Having shown that *Tk* expression was activated specifically by *A. pomorum* in the midgut, but not by *L. brevis*, we focussed specifically on *A. pomorum* in subsequent experiments.

## Intestine-directed *Tk* knockdown abrogates lifespan impact of *A. pomorum*

Next we asked whether *Tk* knockdown in the gut blocked the shortened lifespan of *Ap*-flies, using the *Gal4-UAS* system. In the midgut, EEs are marked by *voila-Gal4*, but this construct is also expressed in some neurons (Lin et al., 2022; Scopelliti et al., 2014), posing a challenge for fully parsing effects of neurons versus midgut EEs. We used a recently-developed intersectional strategy, in which *ChAT-Gal80* represses neuronal *voila-Gal4* (Medina et al., 2024), directing Gal4 to the midgut while sparing CNS. After confirming that this system was effective in our lab's genetic background (Figure S8), we tested whether intestine-directed $Tk^{RNAi}$ abrogated impact of *A. pomorum* on lifespan, including every possible combination of *Voila-Gal4*, *ChAT-Gal80*, and *UAS-*$Tk^{RNAi}$ in a fully-factorial design (Figure 5A-B). Without $Tk^{RNAi}$, lifespan was reduced in *Ap*-flies relative to axenic controls. However, expressing $Tk^{RNAi}$ in all *voila*+ cells (without *ChAT-Gal80*) blocked this effect of *A. pomorum* on lifespan. *ChAT-Gal80* restored a small effect of microbiota, but the magnitude of effect (Z-score) was diminutive, only ~27-40% of that observed in controls. A second CoxPH analysis of the same data confirmed an overall 4-way interaction of *A. pomorum* and the three transgenes, confirming that the impact of *A. pomorum* was contingent on activation of $Tk^{RNAi}$, and the tissues in which it was activated (p=0.0005). These analyses indicated that *Tk* in *Voila*+ cells was obligately required for *A. pomorum* to modulate lifespan, and that the majority of this effect was explained by the intestine.

For further validation we conducted a second lifespan experiment, using independent genetic tools (Rewitz et al., 2024) to direct $Tk^{RNAi}$ specifically to *Tk*+ EEs, with *Gal4* expressed from the *Tk* locus (*Tk-T2A-Gal4*), recombined with a transgene expressing *Gal80* under control of a fragment of *nSyb* promoter (*R57C10-Gal80*), which is expected to lead to pan-neuronal *Gal80* expression as another means to attenuate neuronal Gal4 activity (Kubrak et al., 2022; Malita et al., 2022; Rewitz et al., 2024). We again detected an *A. pomorum*-by-genotype effect (Figure 5C-D), with the lifespan-shortening effect of *A. pomorum* attenuated by *Tk* knockdown, and post-hoc tests showing only a marginal effect of *A. pomorum* on lifespan upon $Tk^{RNAi}$ induction (p=0.08). Independent *Gal4* and *Gal80* transgenes therefore corroborated the finding that intestinal *Tk* is required for the majority of *A. pomorum*'s lifespan-shortening effect.

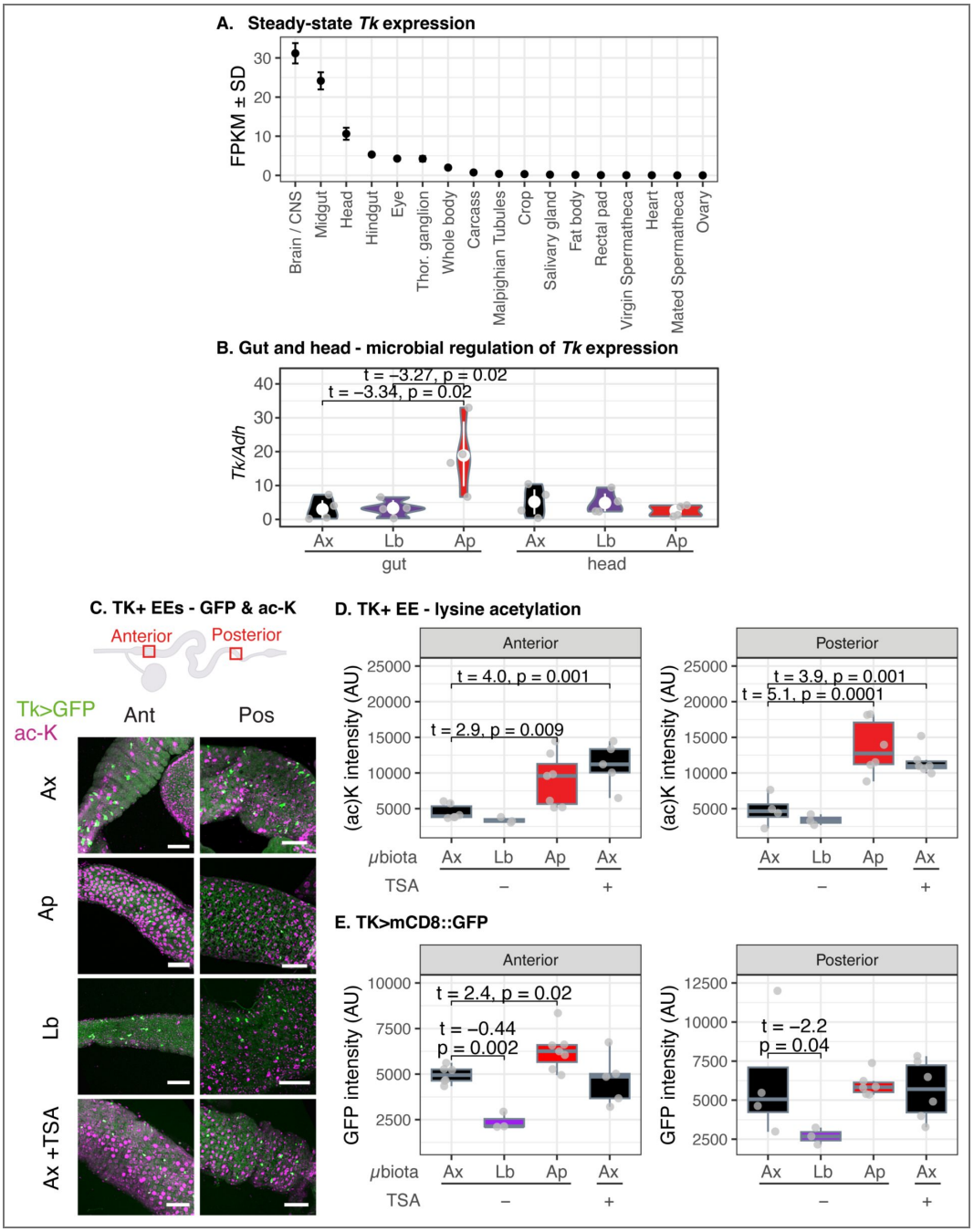



**Figure 4.** ***A. pomorum*** **modulates intestinal** ***Tk*** **expression.**

**A.** *Tk* expression is enriched in nervous system and midgut tissues (data from FlyAtlas2). **B.** RT-qPCR of *Tk* in dissected midguts and heads of axenic and gnotobiotic flies indicates that expression is increased specifically in the midgut by *A. pomorum,* but not in the head; while *L. brevis* affects expression in neither tissue. White point and error bars show mean±SD. Statistics on brackets are from *post-hoc* EMM analyses of linear models, when significant differences were detected. **C.** Quantifying lysine acetylation and *Tk* promoter activity in *Tk*+ cells. Cartoon shows regions quantified in *Tk-Gal4/UAS-mCD8::GFP* flies with acetyl-lysine staining. Scale bars=50μm. **D.** Lysine acetylation is increased specifically by *A. pomorum* in *Tk*+ enteroendocrine cells. Each point represents average fluorescence intensity for acetyl-lysine staining of ≥3 cells (marked by *Tk>GFP*) per gut, in anterior and posterior regions. The HDAC inhibitor Trichostatin-A was fed to axenic flies to confirm that increased histone acetylation is detectable by total acetyl-lysine staining. Statistics from one-way ANOVA, showing comparisons relative to axenic control condition. **E.** *A. pomorum* increases *Tk* promoter activity (*Tk-T2A-Gal4>UAS-GFP*) in anterior but not posterior midgut. Each point represents average GFP intensity of ≥3 cells per gut, in anterior and posterior regions. Statistics from one-way ANOVA, showing comparisons relative to axenic control condition.

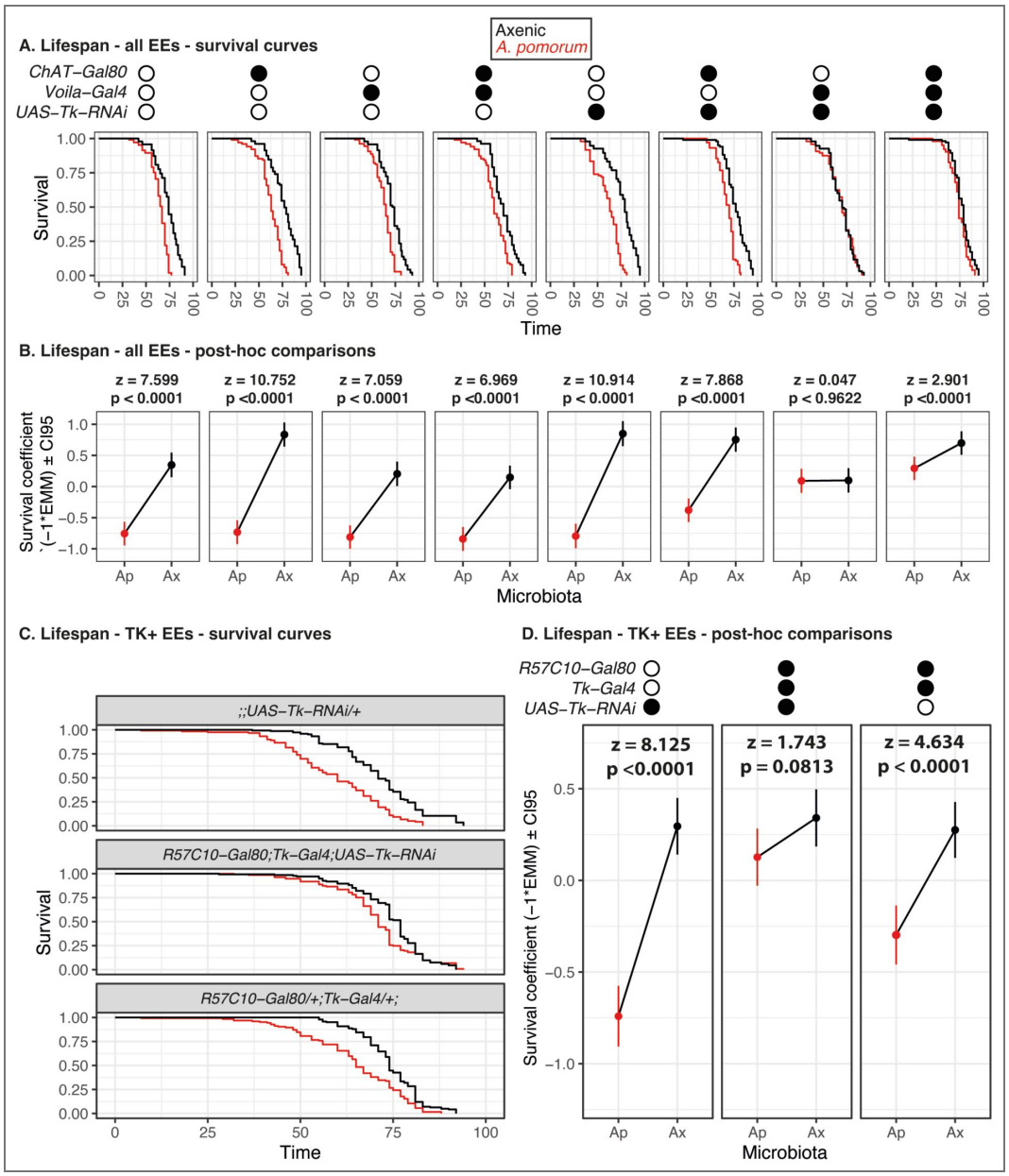



**Figure 5. Gut-directed $Tk^{RNAi}$ abrogates lifespan shortening by *A. pomorum*.**

**A.** Kaplan-Meier survival plots of the indicated genotypes in presence/absence of *A. pomorum*, with knockdown activated by *Voila-Gal4,* in presence/absence of *ChAT-Gal80*. Statistics from *post-hoc* tests of CoxPH model. Genotypes indicated above panels. See Figure S8 for tissue-specificity of transgene combinations. All conditions n=105. **B.** *Post-hoc* analysis of survival model reveals attenuated lifespan response to *A. pomorum* with midgut-directed $Tk^{RNAi}$ (Genotypes as in A). Text indicates differences between *A. pomorum* and axenic flies in the given genotype. CoxPH analyses detected significant interactions both for microbiota*genotype (p<2.2e-16) and for Microbiota:*Voila-Gal4:ChAT-Gal80:UAS-TkRNAi* (p=0.0005). **C.** Kaplan-Meier survival plots of the indicated genotypes in presence/absence of *A. pomorum*, with knockdown activated by *Tk-T2A-Gal4,* and neuronal activity suppressed by *R57C10-Gal80*. **D.** *Post-hoc* analysis of survival model reveals attenuated lifespan response to *A. pomorum* with $Tk^{RNAi}$ directed to midgut by *Tk-T2A -Gal4* and *R57C10-Gal80*. Statistics from *post-hoc* tests of CoxPH model, indicating differences between *Ap*-flies and axenic flies in the given genotype. CoxPH detected overall genotype-by- *A. pomorum* interaction (p=2.46e-5).

## Role for neuronal *TkR99D*$^{RNAi}$ in host ageing effects of *A. pomorum*

We asked which receptors might mediate the *Tk*-dependent response to *A. pomorum*, and from which tissues. The fly genome expresses two cognate Tk receptors (TkRs) - *TkR86C* and *TkR99D* - which have different affinities for the six peptides encoded by *Tk* (Nässel et al., 2019), and distinct physiological roles. Plotting steady-state *TkR86C* and *TkR99D* expression, again using FlyAtlas2 (CR fly) data (Leader et al., 2018), revealed that both TkRs were most strongly expressed in CNS (Figure 6A). We expected that knocking down either one or both TkRs should extend lifespan in *Ap*-flies, mimicking effects of *Tk* knockdown.

We tested the lifespan impacts of adult-onset TkR manipulation. RNAi constructs were developmentally lethal when crossed to ubiquitous drivers (*DaGS* and *ActGS*), even in the absence of inducer (RU), so instead we established lifespan experiments using the pan-neuronal driver, *Elav-GS*. Lifespan was shorter in *Ap*-flies relative to controls, and RNAi against each TkR interacted with the lifespan effect of *A. pomorum*, but in notably distinct ways. *TkR86C*$^{RNAi}$ had no effect in *Ap*-flies, but axenics were no longer long-lived (Figure 6B-C): this reveals an interaction with *A. pomorum* but does not recapitulate that of *Tk*$^{RNAi}$, suggesting that TkR86C likely does not signal downstream of the *A. pomorum-Tk* relay. By contrast, *TkR99D*$^{RNAi}$ extended lifespan in *Ap*-flies, equivalent to axenics (Figure 6D-E). The most parsimonious explanation of these results is an *Acetobacter*-Tk-TkR99D relay, which shortens lifespan.

In fly midgut, TkR99D reportedly plays a role in mobilising lipid stores in enterocytes of the fly midgut (Song et al., 2014), prompting us to ask whether it may play an additional role in lifespan from these cells. However, expressing *TkR99D*$^{RNAi}$ with the enterocyte driver *Mex-GS* did not extend *Ap*-flies lifespan, suggesting that the *A. pomorum*-Tk relay signals gut-nonautonomously (Figure S9).

Finally, we asked whether lifespan extension in axenics and upon knockdown of *TkR99D* was accompanied by improved health in older flies (i.e. healthspan). Fly microbiota compromise healthspan by promoting gut barrier dysfunction in aged animals, characterised by permeability which shortly precedes death (Clark et al., 2015; Regan et al., 2016; Rera et al., 2012; Zane et al., 2023). We therefore tested whether *A. pomorum* promoted gut barrier dysfunction, and whether this could be rescued by *TkR99D*$^{RNAi}$ induction. We aged axenic and *Ap*-flies, with *TkR99D*$^{RNAi}$ from day 3 of adulthood onwards, before feeding on a blue dye to report gut permeability after 56 days (Figure 6F). *Ap*-flies indeed exhibited a higher rate of gut permeability than axenics, but *TkR99D*$^{RNAi}$ rescued the pathology to a level that was not significantly different from axenics. Thus, *TkR99D*$^{RNAi}$ can ameliorate deleterious impacts of *A. pomorum* on late-life health, suggesting that the putative *A. pomorum-Tk-TkR99D* relay limits late-life health.

## Lifespan and metabolic impacts of *A. pomorum* are not explained by *TkR99D* in insulin-producing cells

What happens downstream of the putative *A. pomorum*-Tk-TkR99D relay? Reduced insulin signalling promotes healthy ageing across species, with lifespan and other benefits reported in flies, nematodes and rodents, and genetic associations indicating potential conservation in humans (Clancy et al., 2001; Kenyon et al., 1993; Selman et al., 2008; Slagboom et al., 2017). *A. pomorum* is reported to activate insulin signalling (S. Shin et al., 2011), while *TkR99D* expression is reported in insulin-producing cells (IPCs) in the fly brain (Birse et al., 2011), ablation of which extends lifespan (Alic et al., 2014; Broughton et al., 2005). We therefore hypothesised that Tk-TkR99D signalling impacts ageing by serving as a relay between the gut microbiota and IPCs (Figure 7A).

We tested whether insulin signalling was required for lifespan to respond to *A. pomorum*. The forkhead box-O transcription factor dFOXO, encoded by the *dFoxO* locus, is obligately required for longevity upon reduced insulin signalling (Slack et al., 2015, 2011). dFOXO is retained in cytoplasm of Ap-flies, consistent with activated insulin signalling (S. Shin et al., 2011). We

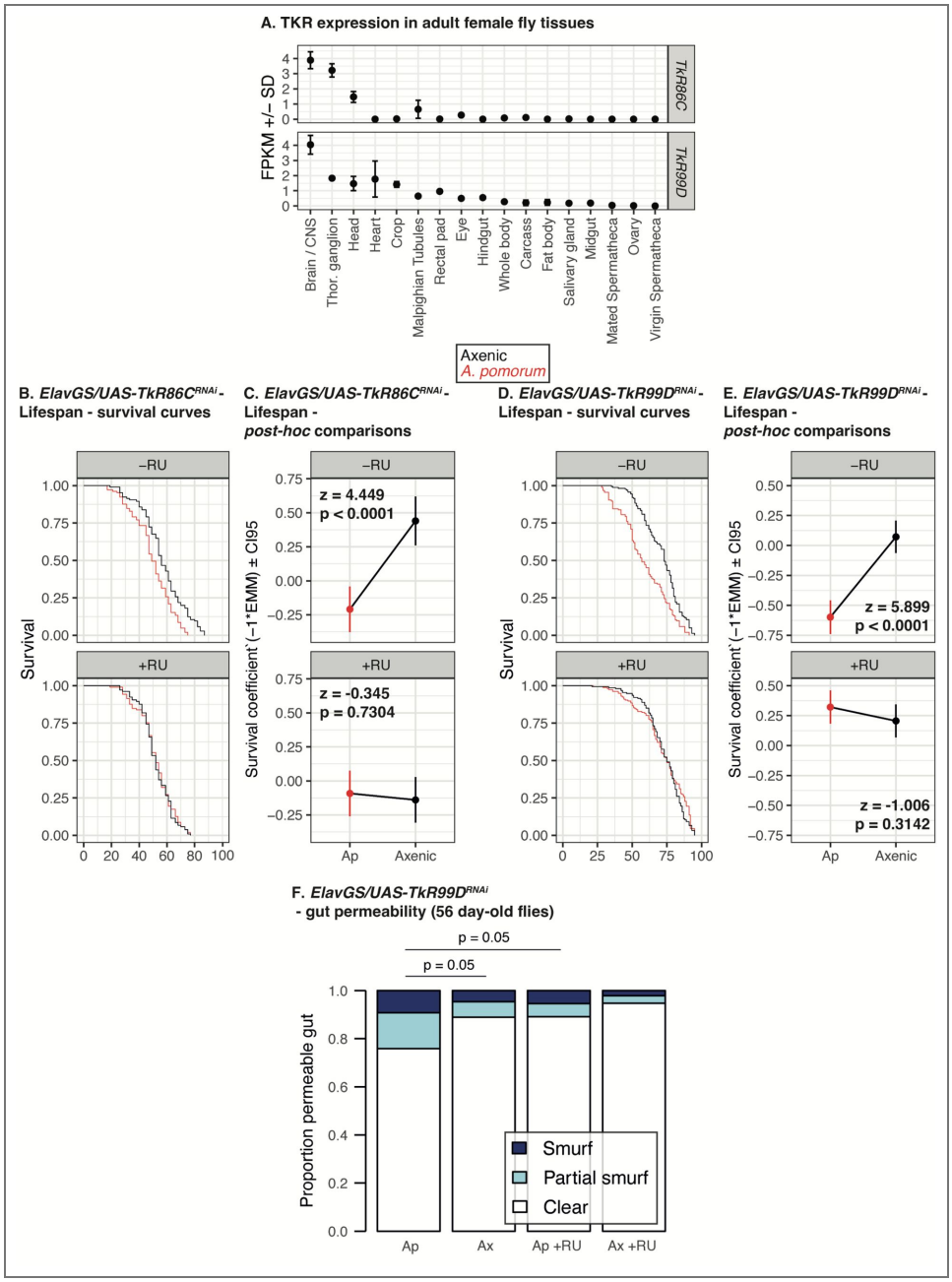



**Figure 6. Pan-neuronal *TkR99D*$^{RNAi}$ abrogates lifespan impact of *A. pomorum*.**

**A.** FlyAtlas data showing expression of the two cognate *Drosophila* Tk receptors, *TkR86C* and *TkR99D*, among adult female tissues. **B.** Kaplan-Meier survival plots of *Elav-GS; UAS-TkR86C*$^{RNAi}$ flies in presence/absence of *A. pomorum*, with knockdown activated by RU486 feeding (bottom facet). CoxPH detected significant microbiota*RU interaction (p=0.0005). All conditions n=105. **C.** Post-hoc analysis of data from B showing that *TkR86C*$^{RNAi}$ induction locks axenic flies into a shortened lifespan. Text indicates differences between *A. pomorum* and axenic flies in each given RU condition. **D.** Kaplan-Meier survival plots of *Elav-GeneSwitch; UAS-TkR99D*$^{RNAi}$ flies in presence/absence of *A. pomorum*, with knockdown activated by RU486 feeding (bottom facet). CoxPH detected significant microbiota*RU interaction (p=1.362e-6). Ap RU- n=163, Ap RU+ n=161, Axenic RU- n=165, Axenic RU+ n=164 (pool of two replicate experiments). **E.** Post-hoc analysis of data from B showing that *TkR99D*$^{RNAi}$ induction blocks shortening of lifespan by *A. pomorum*. Text indicates differences between *A. pomorum* and axenic flies in each given RU condition. **F.** Neuronal *TkR99D*$^{RNAi}$ blocks onset of gut permeability ("smurf" phenotype) in aged flies. Bars show relative frequencies of flies with blue dye appearing to permeate through all tissues ("full smurf") and through abdomen/thorax ("partial smurf"). $\chi^2$=16.126, DF=6, p=0.013. P-values on figure from chi-square tests of each given pair of columns, shown when differences were observed (otherwise omitted).

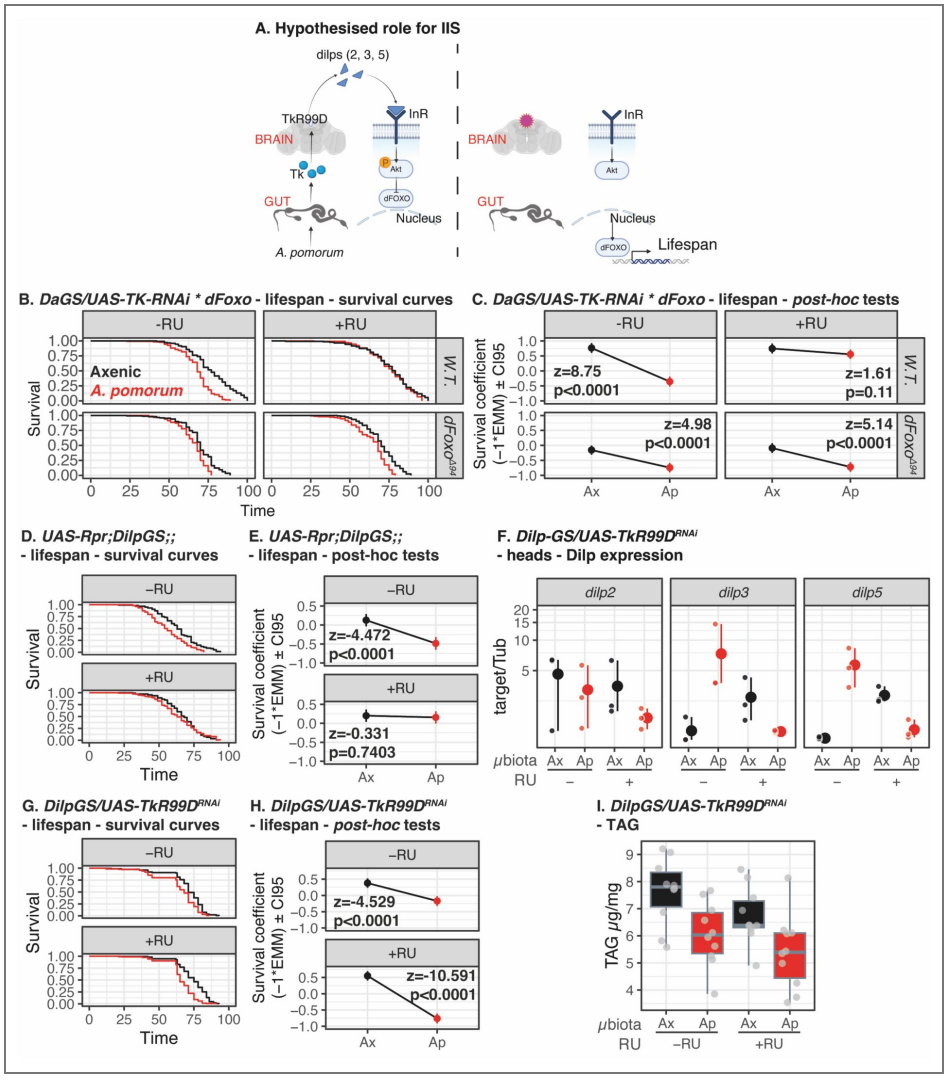



**Figure 7. Insulin signalling is involved in lifespan response to *A. pomorum* and *TkR99D* knockdown, but not obligately required.**

**A.** Hypothetical model of role for insulin signalling. In presence of microbiota (left), Tk peptides are released from gut and contact TkR99D in IPCs/brain. IPCs are released into circulation, activating insulin signalling in peripheral tissues and leading to nuclear exclusion of dFOXO. In absence of microbiota (right), extracellular signalling is diminished, reducing downstream insulin signalling and consequent activation of lifespan-extending gene expression program by dFOXO. **B.** dFOXO connects lifespan responses to *A. pomorum* and ubiquitous $Tk^{RNAi}$. Facets show Kaplan-Meier plots in axenic and *A. pomorum* conditions, with/without RNAi induction (RU), and with/without $dFoxO^{\Delta 94}$ null mutation. Overall microbiota*RU*Foxo interaction p=5.384e-05 (CoxPH). All conditions n=150 except W.T. *A. pomorum* -RU (n=135) and W.T. axenic +RU (n=148). **C.** Post-hoc comparisons (EMmeans), showing impact of *A. pomorum* under specified conditions of *TkRNAi* induction and *dFoxO* deletion. *A. pomorum* shortens wild-type lifespan, but this effect is diminished by $dFoxO^{\Delta 94}$. $Tk^{RNAi}$ induction blocks lifespan shortening by *A. pomorum* in wild-type background, but not in $dFoxO^{\Delta 94}$ background; altogether suggesting that *dFoxO* is required for *A. pomorum* to influence longevity via Tk modulation. **D-E.** Insulin-producing cells (IPCs) are required for *A. pomorum* to influence lifespan (CoxPH p=0.003). All conditions n=120. Post-hoc tests (**E**) confirm that lifespan effect of *A. pomorum* (-RU) is absent following IPC ablation (*Rpr* expression, +RU). **F.** *A. pomorum* increases expression of *Dilp3* and *Dilp5,* but not *Dilp2*, in a *TkR99D* -dependent manner. Panels show expression (RT-qPCR) in heads of axenic and *Ap*-flies ±$TkR99D^{RNAi}$ in IPCs (*Dilp2-GS/UAS-*$TkR99D^{RNAi}$). by Microbiota*RU interactions were tested for with linear models: *Dilp2* $F_{1,24}$=0.424, p=0.5, *Dilp3* $F_{1,24}$=23.404, p=0.0001, *Dilp5* $F_{1,24}$=29.261, p<0.0001. **G-H.** $TkR99D^{RNAi}$ in IPCs worsens lifespan impact of *A. pomorum*. Ap -RU n=160, Ap +RU n=162, Ax -RU n=160, Ax +RU n=163. (**I**) Post-hoc tests indicate accentuated shortening of lifespan by *A. pomorum* following $TkR99D^{RNAi}$ induction (+RU). **I.** $TkR99D^{RNAi}$ in IPCs does not modulate metabolic impact of *A. pomorum* on fly TAG. The two experimental factors each had significant effects on TAG (ANOVA, *A. pomorum* F=14.95, Df=1,37, p=0.0004; RU F=4.32, Df=1,37, p=0.04), but no interaction was detected.

recombined a dFOXO null mutation ($dFoxO^{\Delta 94}$) with transgenes to knock down *Tk* ubiquitously, performed lifespan assays, and used Cox proportional hazards analysis to test for an *A. pomorum*Tk*dFoxO* interaction (Figure 7B-C). We observed a significant three-way interaction (CoxPH p=5.384e-05) – indicating that $dFoxO^{\Delta 94}$ modulated the microbiota-*Tk* interaction – and then used post-hoc tests to dissect the co-dependence of the three experimental variables (Table 1). In controls without *Tk* knockdown, *A. pomorum* shortened lifespan relative to axenics in wild-type flies, but $dFoxO^{\Delta 94}$ flies exhibited a diminished but still-significant shortening of lifespan by *A. pomorum*. Without $dFoxO^{\Delta 94}$, *Tk* knockdown blocked lifespan response by rendering *Ap*-flies long-lived. In the $dFoxO^{\Delta 94}$ background, *Tk* knockdown did not extend lifespan, in either *Ap*-flies or axenics. However, unexpectedly, $Tk^{RNAi}$ ceased to block the effect of *A. pomorum* in the $dFoxO^{\Delta 94}$ background. Thus, in summary, (A) *dFoxO* is partially required for lifespan effect of *A. pomorum*, (B) *dFoxO* is required for lifespan extension upon *Tk* knockdown, and (C) *dFoxO* is required for *A. pomorum* and *Tk* knockdown to have an interactive effect on lifespan. We interpret that *dFoxO* potentiates the putative *A. pomorum*-Tk-TkR99D relay.

To assess directly the role of tissues reported to express *TkR99D* (Birse et al., 2011), we turned to the IPCs. We first tested whether ablating these cells by expressing the pro-apoptotic factor *Rpr* would block lifespan response to *A. pomorum*, reasoning that a role for these cells in response to the bacteria would be shown by a lack of response following ablation. Lifespan was again shortened in *Ap*-flies relative to axenic controls. However this effect was blocked by adult-onset *Rpr* expression in IPCs (using the *Dilp2-GS* driver) (Figure 7D-E), and we detected an overall microbiota*RU interaction (CoxPH p=0.003). This indicated a role for IPCs in lifespan shortening by *A. pomorum*. To test whether TkR99D activation could account for this effect we expressed $TkR99D^{RNAi}$ in IPCs, and assayed expression of the three insulin-like peptides expressed therein (*Ilp2, Ilp3, Ilp5*) by RT-qPCR of heads (Figure 7F, Table 2). *Ilp2* exhibited no response to *A. pomorum* or $TkR99D^{RNAi}$.(linear model, microbiota:RU interaction $F_{1,24}$=0.424, p=0.5) However, for *Ilp3* and *Ilp5*, *A. pomorum* promoted expression, and this effect was blocked by $TkR99D^{RNAi}$ induction (linear models, microbiota:RU interactions *Ilp3* $F_{1,24}$=23.404, p=0.0001, *Ilp5* $F_{1,24}$=29.261, p<0.0001.) Specifically, *Ilp3* and *Ilp5* expression levels were increased in *Ap*-flies, but these effects were absent upon $TkR99D^{RNAi}$ induction (Table 2).

Finally, we tested whether $TkR99D^{RNAi}$ in IPCs blocked lifespan shortening by *A. pomorum* (Figures 7G-H). As expected, without $TkR99D^{RNAi}$, *Ap*-flies were shorter-lived than axenics. $TkR99D^{RNAi}$ induction in IPCs did not affect axenic lifespan but, surprisingly, the lifespan-shortening effect of *A. pomorum* was exaggerated by $TkR99D^{RNAi}$ induction (Figure 7H). Consequently, *A. pomorum's* effect on lifespan seemingly cannot be explained by TkR99D activation in IPCs. We also tested whether $TkR99D^{RNAi}$ in IPCs altered effects of *A. pomorum* on TAG levels in young flies (Figure 7I): no interaction was apparent (though we detected independent effects of microbiota (ANOVA F=14.95, Df=1,37, p=0.0004) and RNAi induction (F=4.32, Df=1,37, p=0.04)), suggesting that interactive effects of *A. pomorum* and Tk peptides are not mediated by *TkR99D* in IPCs.

Altogether, these data outline involvement of insulin signalling in responses to *A. pomorum* and *Tk*, because IPC ablation blocked lifespan shortening by *A. pomorum* (Figure 7D-E), and because *dFoxO* was required for the interaction of *A. pomorum* and $Tk^{RNAi}$. However, the failure of $TkR99D^{RNAi}$ in IPCs to block *A. pomorum's* effects on lifespan or TAG suggests that insulin signalling does not act downstream of the putative *A. pomorum*/Tk/TkR signalling relay. These data may suggest priming, with insulin signalling required to potentiate responses to *A. pomorum*/Tk/TkR.

## No evidence of role for *TkR99D* in Akh-producing cells in lifespan and metabolic response to *A. pomorum*

In insects, adipokinetic hormone (Akh) is released from *corpora cardiaca* (cc) to promote lipid mobilisation in response to heightened energy demand, analogous to glucagon in mammals (Gäde and Marco, 2022; Musselman et al., 2018; Toprak et al., 2020). Having observed *Tk*-

**Table 1.** ***dFoxO*** **is partially required for microbiota*** ***Tk*** **interaction for lifespan: post-hoc pairwise comparisons (EMMeans)**

| contrast | estimate | SE | z.ratio | p.value |
|---|---|---|---|---|
| (Ax WT RU-) - (Ap WT RU-) | -1.12 | 0.13 | -8.76 | 6.38E-14 |
| (Ax WT RU-) - (Ax dFoxOΔ RU-) | -0.92 | 0.12 | -7.51 | 1.75E-12 |
| (Ax WT RU-) - (Ap dFoxOΔ RU-) | -1.51 | 0.13 | -11.87 | 0 |
| (Ax WT RU-) - (Ax WT RU+) | -0.02 | 0.12 | -0.15 | 0.9999999 |
| (Ax WT RU-) - (Ap WT RU+) | -0.21 | 0.12 | -1.77 | 0.63954118 |
| (Ax WT RU-) - (Ax dFoxOΔ RU+) | -0.85 | 0.13 | -6.79 | 3.21E-10 |
| (Ax WT RU-) - (Ap dFoxOΔ RU+) | -1.48 | 0.13 | -11.65 | 0 |
| (Ap WT RU-) - (Ax dFoxOΔ RU-) | 0.2 | 0.12 | 1.66 | 0.71153814 |
| (Ap WT RU-) - (Ap dFoxOΔ RU-) | -0.39 | 0.12 | -3.16 | 0.03369588 |
| (Ap WT RU-) - (Ax WT RU+) | 1.1 | 0.13 | 8.6 | 7.16E-14 |
| (Ap WT RU-) - (Ap WT RU+) | 0.91 | 0.13 | 7.27 | 1.00E-11 |
| (Ap WT RU-) - (Ax dFoxOΔ RU+) | 0.27 | 0.13 | 2.13 | 0.39332695 |
| (Ap WT RU-) - (Ap dFoxOΔ RU+) | -0.36 | 0.12 | -2.96 | 0.06098085 |
| (Ax dFoxOΔ RU-) - (Ap dFoxOΔ RU-) | -0.59 | 0.12 | -4.99 | 1.70E-05 |
| (Ax dFoxOΔ RU-) - (Ax WT RU+) | 0.9 | 0.12 | 7.34 | 5.87E-12 |
| (Ax dFoxOΔ RU-) - (Ap WT RU+) | 0.71 | 0.12 | 5.93 | 8.56E-08 |
| (Ax dFoxOΔ RU-) - (Ax dFoxOΔ RU+) | 0.06 | 0.12 | 0.54 | 0.99943704 |
| (Ax dFoxOΔ RU-) - (Ap dFoxOΔ RU+) | -0.57 | 0.12 | -4.77 | 5.07E-05 |
| (Ap dFoxOΔ RU-) - (Ax WT RU+) | 1.49 | 0.13 | 11.7 | 0 |
| (Ap dFoxOΔ RU-) - (Ap WT RU+) | 1.3 | 0.12 | 10.45 | 7.78E-14 |
| (Ap dFoxOΔ RU-) - (Ax dFoxOΔ RU+) | 0.65 | 0.12 | 5.35 | 2.43E-06 |
| (Ap dFoxOΔ RU-) - (Ap dFoxOΔ RU+) | 0.02 | 0.12 | 0.19 | 0.99999961 |
| (Ax WT RU+) - (Ap WT RU+) | -0.19 | 0.12 | -1.61 | 0.74370666 |
| (Ax WT RU+) - (Ax dFoxOΔ RU+) | -0.84 | 0.13 | -6.63 | 9.28E-10 |
| (Ax WT RU+) - (Ap dFoxOΔ RU+) | -1.47 | 0.13 | -11.49 | 0 |
| (Ap WT RU+) - (Ax dFoxOΔ RU+) | -0.64 | 0.12 | -5.23 | 4.63E-06 |
| (Ap WT RU+) - (Ap dFoxOΔ RU+) | -1.27 | 0.12 | -10.23 | 6.75E-14 |
| (Ax dFoxOΔ RU+) - (Ap dFoxOΔ RU+) | -0.63 | 0.12 | -5.14 | 7.52E-06 |

**Table 2.** ***TkR99D*** $^{RNAi}$ **in insulin-producing cells blocks increased expression of** ***dilp3*** **and** ***dilp5*****, but not** ***dilp2*****, by** ***A. pomorum*****:** ***post-hoc*** **tests.**

| Transcript | contrast | estimate | SE | df | t.ratio | p.value |
|---|---|---|---|---|---|---|
| dilp2 | (axenic -) - (ap -) | 0.34 | 0.82 | 24 | 0.42 | 0.98 |
| | (axenic -) - (axenic +) | 0.05 | 0.82 | 24 | 0.06 | 1 |
| | (axenic -) - (ap +) | 1.15 | 0.82 | 24 | 1.4 | 0.51 |
| | (ap -) - (axenic +) | -0.29 | 0.82 | 24 | -0.35 | 0.98 |
| | (ap -) - (ap +) | 0.81 | 0.82 | 24 | 0.98 | 0.76 |
| | (axenic +) - (ap +) | 1.1 | 0.82 | 24 | 1.34 | 0.55 |
| dilp3 | (axenic -) - (ap -) | -3.9 | 0.82 | 24 | -4.74 | 0.0004 |
| | (axenic -) - (axenic +) | -2.67 | 0.82 | 24 | -3.24 | 0.02 |
| | (axenic -) - (ap +) | -0.93 | 0.82 | 24 | -1.13 | 0.67 |
| | (ap -) - (axenic +) | 1.23 | 0.82 | 24 | 1.5 | 0.45 |
| | (ap -) - (ap +) | 2.97 | 0.82 | 24 | 3.6 | 0.01 |
| | (axenic +) - (ap +) | 1.73 | 0.82 | 24 | 2.1 | 0.18 |
| dilp5 | (axenic -) - (ap -) | -4.18 | 0.82 | 24 | -5.07 | 0.0002 |
| | (axenic -) - (axenic +) | -3.32 | 0.82 | 24 | -4.03 | 0.003 |
| | (axenic -) - (ap +) | -1.19 | 0.82 | 24 | -1.45 | 0.48 |
| | (ap -) - (axenic +) | 0.86 | 0.82 | 24 | 1.04 | 0.73 |
| | (ap -) - (ap +) | 2.98 | 0.82 | 24 | 3.62 | 0.01 |
| | (axenic +) - (ap +) | 2.13 | 0.82 | 24 | 2.58 | 0.07 |

dependent effects of microbiota on TAG storage, and following recent evidence that *TkR99D* in Akh-producing cells limits lifespan (Ahrentløv et al., 2025), we tested whether it may also mediate lifespan impact of *A. pomorum*.

First, to test whether Akh peptide levels in CC were *A. pomorum*-dependent, we quantified Akh with antibody staining. Akh staining levels were significantly higher in CC dissected from *Ap*-flies than axenics (Figure 8A), suggesting microbial regulation of Akh signalling.

We then tested directly whether TkR99D was required in Akh-producing cells for *A. pomorum* to shorten lifespan. *A. pomorum* shortened lifespan across the all different *Akh* conditions (Figure 8B-C). Expressing $TkR99D^{RNAi}$ under control of *Akh-Gal4* did extend lifespan in both axenics and *Ap*-flies, corroborating a recent report of the CC's role in longevity (Ahrentløv et al., 2025) (noting background effects, despite backcrossing immediately before experimentation) (Figure 8B-C). However, these data indicate that lifespan shortening by *A. pomorum* is independent of TkR99D in CC. We also tested whether RNAi against Akh receptor (*AkhR*) in adipose (fat body) altered response to *A. pomorum*, driving expression with *S106-GS*, but again no interaction was apparent (Figure S10). Finally, we tested whether *TkR99D* in Akh-producing cells interacted with effects of *A. pomorum* on TAG storage. $TkR99D^{RNAi}$ induction did not block response to *A. pomorum*, with *Ap*-flies storing less lipid than axenics independent of fly genotype (Figure 8D), indicating that *A. pomorum*'s influence on TAG does not depend on TkR99D in CC. Combined with our investigation of insulin signalling, our studies of Akh suggest that the impact of *Acetobacter* on ageing is mediated by non-canonical mechanisms that depend on *Tk* and *TkR99D*.

# Discussion

Microbiota impact ageing across animals (Cabreiro et al., 2013; Cui et al., 2019; Sannino et al., 2018; Seidel and Valenzano, 2018; Smith et al., 2017; Snyder et al., 1990; Tazume et al., 1991), and connections between gut and the nervous system are key to health throughout the lifecourse (Boehme et al., 2022; Dinan and Cryan, 2017; Qansuwa et al., 2024). Here we have connected microbial regulation of ageing with tissue-specific regulation of endocrine signalling genes in gut and neurons. Our *Drosophila* results show that (A) *A. pomorum* is sufficient to modulate *Tk* in the midgut, (B) knocking down the peptide in midgut blocks the lifespan response to the bacteria, and (C) neuronal knockdown of the receptor *TkR99D* recapitulates these effects. We are struck by the specificity of this circuit, with knockdown of a single peptide-encoding locus in a specific tissue ablating the microbial impact on lifespan, despite the wide array of host functions that the microbiota affect. This specific requirement can likely be explained by the highly pleiotropic functions of Tachykinins (Nässel, 2025; Nässel et al., 2019), suggesting that *Tk* knockdown reprograms organismal physiology to a state in which lifespan can no longer be modulated by microbiota. Given the evolutionary conservation of tachykinins, it will be interesting in future work to ask whether this family of peptides can also modulate ageing in other species.

Our work indicates that the influence of the microbiota-*Tk/TkR99D* relay is partially independent of better-studied lifespan assurance mechanisms, i.e. insulin signalling, and phenotypes associated with ageing. The lifespan benefit of *Tk* knockdown was not accompanied by costs to fecundity or feeding rate in CR flies, but instead knockdown altered metabolic impact of microbiota. Furthermore, knocking down *TkR99D* in major signalling hubs associated with ageing and TAG metabolism (IPCs and Akh+ cells, respectively) did not block lifespan or TAG response to *A. pomorum*, suggesting that yet-uncharacterised neuroendocrine mechanisms are at play. We interpret the partial requirement for *dFoxO*, connecting *A. pomorum* to $Tk^{RNAi}$ modulation of lifespan, as suggesting that insulin potentiates downstream responsiveness, without necessarily being involved directly by *TkR99D* in IPCs.

Our work was performed entirely in females. We chose to study the sex that tends to exhibit more robust response to anti-ageing interventions (Regan and Partridge, 2013), because of the labour-intensive and technically challenging nature of performing lifespan experiments under lifelong

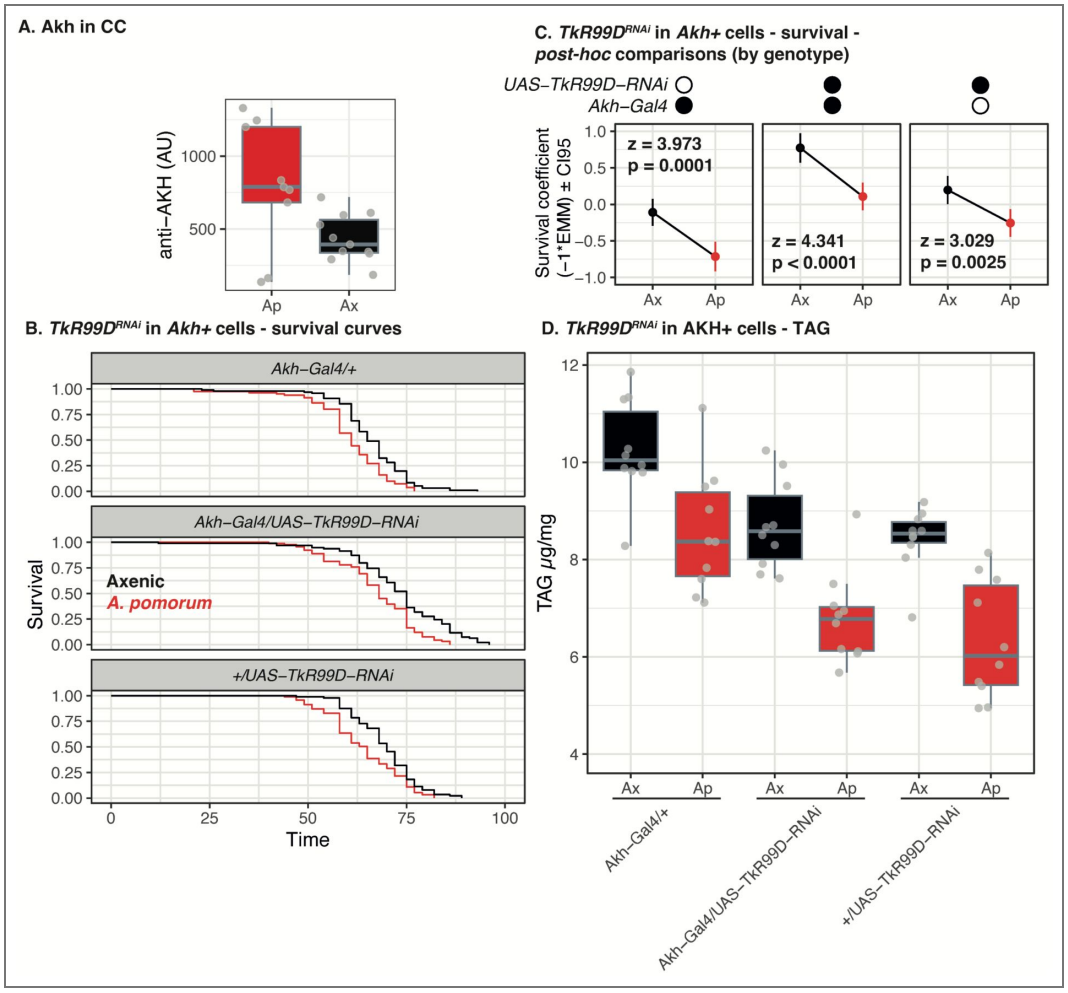



**Figure 8.** ***A. pomorum*** **modulation of host lifespan and TAG is independent of** ***TkR99D*** **in Akh-producing cells.**

**A.** Antibody staining of Akh in dissected Corpora Cardiaca (CC) is increased in *Ap*-flies (Ap) relative to axenics (Ax). (t-test: t = −2.36, p-value = 0.04). **B.** Microbial shortening of lifespan is independent of *TkR99D* signalling in Akh+ cells. Facets show Kaplan-Meier survival curves for axenic and *Ap*-flies when *TkR99D* is knocked down (middle facet) and in each control genotype. No significant interaction of genotype and *A. pomorum* was detected (CoxPH p=0.58). *Akh-Gal4/+ A. pomorum* n=103, *Akh-Gal4/+* axenic n=108, *Akh-Gal4/UAS-TkR99D-RNAi A. pomorum* n=105, *Akh-Gal4/UAS-TkR99D-RNAi* axenic n=107, *+/UAS-TkR99D-RNAi A. pomorum* n=103, *+/UAS-TkR99D-RNAi* axenic n=101. **C.** Post-hoc comparisons (EMmeans) confirm equivalent lifespan shortening by *A. pomorum*, independent of *TkR99D*$^{RNAi}$. Combination of transgenes indicated above facets. **D.** *A. pomorum* modulation of host TAG is independent of *TkR99D*$^{RNAi}$ in Akh+ cells. Boxplots show TAG levels are reduced independent of *TkR99D*$^{RNAi}$ induction. No significant interaction of genotype and *A. pomorum* detected.

axenic conditions. Having established this the *A. pomorum-Tk-TkR99D* connection, it will be interesting in future work to ask whether males also enjoy a lifespan benefit of diminishing the activity of this circuit.

*Tk* is one of a number of signalling circuits implicated downstream of the microbiota, for example JAK-STAT and JNK signalling (Buchon et al., 2009), IMD (Jugder et al., 2021; Watnick and Jugder, 2020), and the neuropeptide CNMamide (Kim et al., 2021). We do not propose that Tachykinins alone are sufficient to explain all physiological effects of the microbiota: rather we expect that the interplay of multiple signalling pathways collectively orchestrate the host organismal response to microbiota. Indeed our transcriptome (re)analysis (Figure 1B) suggested that a community of bacteria co-regulate expression of the neuropeptides *ITP*, *AstC* and *NPF* alongside *Tk*. We note that *Tk* and *NPF* are co-expressed in a number of the same EE populations (Guo et al., 2019), and related functions on nutrient-specific appetite, physiology and Akh signalling are reported (Ahrentløv et al., 2025; Malita et al., 2022), suggesting closely coupled functions for Tk and NPF which may also be relevant to integrating responses to microbiota. Further work should aim to identify the broader signalling networks that respond to the microbiota, and the extent to which the constituents and structure of those networks mirror what is known in other contexts (e.g. responses to diet or pathogenic infection).

A key element to better understanding how signalling networks at large respond to microbiota will be to better understand what properties of the microbiota the signals convey. Signalling is selected to convey information about environmental, nutritional, and physiological state: which of these parameters do the microbiota impact? Impacts on metabolism could be mediated by direct provision of nutrients (e.g. short-chain fatty acids, B-vitamins (Sannino et al., 2018; S. C. Shin et al., 2011; A. C.-N. Wong et al., 2014)), and/or competition with the host by removal of nutrients from diet (Huang and Douglas, 2015). Immunity may be modulated by bacterial titre, to prevent pathological overgrowth of otherwise mutualistic bacteria. Our results indicate that the interplay between bacteria and *Tk* knockdown is bacteria-specific, suggesting in turn that more complex mixes of bacteria that co-occur with the fly may elicit distinct signalling network topologies. Furthermore, the previous finding that fly transcriptional networks are structured by microbiota (Dobson et al., 2016) leads to a question of whether other host networks - such as endocrine signalling - may also be structured by microbiota, in which case signalling in the presence of one bacterium might elicit quite different physiological responses than in the presence of a distinct bacterium.

The two biggest mechanistic questions arising from this work are (A) from where in the nervous system does *TkR99D* inhibition extend lifespan? and (B) by what mechanism does inhibition extend lifespan? We tested two neuroendocrine tissues, the IPCs and the Akh+ cells, finding that $TkR99D^{RNAi}$ in either did not block shortened lifespan or TAG phenotypes in *Ap*-flies (though bacteria-independent effects of $TkR99D^{RNAi}$ in *Akh*+ cells were apparent). These data suggest that we can reject a role for TkR99D in IPCs and *Akh*+ cells, and instead that an elusive population of *TkR99D*+ neurons is at play downstream of the *Acetobacter-Tk* relay. With respect to future mechanistic understanding, we suggest it will be important to note that pan-neuronal $TkR99D^{RNAi}$ also mitigated gut permeability (smurfing) in aged *Ap*-fies, because the smurf phenotype is underpinned by increased immune system activity (Clark et al., 2015; Clark and Walker, 2017), and activation of *Tk* cells by *Acetobacter* is dependent on immune (IMD) signalling (Jugder et al., 2021). Together these observations suggest close links between *Tk* signalling and immunity, outlining a possible role for *Acetobacter/Tk/TkR99D* in “inflammaging”.

The biggest comparative question arising from our work is whether our findings are relevant in other animals, potentially including mammals. Flies have a track record of revealing mechanisms of ageing that are conserved in other systems (Bjedov et al., 2010; Clancy et al., 2001; Harrison et al., 2009; Kenyon et al., 1993; Miller et al., 2014; Selman et al., 2008; Wilkinson et al., 2012; Zhang et al., 2024). Tachykinins are conserved from invertebrates to mammals, raising the question of whether their functions in ageing are also conserved. The next step would most likely be to assess whether Tachykinin knockdown has anti-ageing effects in rodents, and/or short-lived vertebrates like the African turquoise killifish. Such studies may pave the way for drug

repurposing, as some small-molecule inhibitors of human TkRs have already been developed and are licenced in some jurisdictions for specific purposes. Such interest is timely since the therapeutic role of TkRs is receiving renewed attention for potential metabolic therapies (Sass et al., 2024).

In conclusion, our study establishes a mechanistic connection between the microbiota and ageing via gut/neuron signalling. Microbial shortening of host lifespan depends on *Tk/TkR* signalling, which can be explained by *Acetobacter* activating intestinal *Tk* and, downstream, neuronal *TkR99D*. The findings hint at effects which may be conserved in other systems, which may ultimately help to explain mechanistically why microbiota exert a conserved influence on animal health and ageing.

# Materials and methods

## Flies, bacteria, husbandry and culturing

Stocks used are detailed in Table 3. All stocks (except for recombinant *R57C10-Gal80;;Tk-T2A-Gal4*, gift from Kim Rewitz) were backcrossed into the Dahomey background (originally isolated from Benin in the 1970s), bearing the *w1118* mutation and confirmed *Wolbachia*-negative. Presence of $dFoxO^{\Delta 94}$ in recombinant flies was confirmed by PCR of mothers of single-fly crosses.

All flies were maintained at 25°C with a 12-12-hour light-dark cycle on SYA medium, comprising 5% sucrose (MP Biomedicals BP220), 10% yeast (MP Biomedicals, 903312, lot no. S7760), and 1.5% agar (Sigma-Aldrich A7002)(Bass et al., 2007; Sannino and Dobson, 2023). Preservatives (0.3% nipagin, 0.3% propionic acid) were incorporated into media for stock maintenance. Crosses were performed on juice agar in egg laying cages (per litre: 20g agar, 26g sucrose, 52g glucose, 7g yeast, 88.8ml organic clementine or grape juice). To generate axenic flies, eggs were collected and washed 2x in alternating 10% bleach for (3-5 min) and sterile ddH2O for (1 min), with an optional additional 1 min wash in EtOH, before being allowed to pellet by gravity in sterile PBS. 20 µl of egg pellet was aspirated with wide-mouthed pipette tips into T75 flasks containing 50mL SYA. Preservatives were included during fly development only in CR experiments. We made gnotobiotic embryos as as previously (Sannino and Dobson, 2023). M9+lactate media comprised 200ml 5x M9 salts, 2ml 1M $MgSO_4$, 1ml 1M $CaCl_2$, 25ml 20% (v/v) lactate, made up to 1l in $ddH_2O$ and autoclaved. YPD media comprised 10g yeast extract (VWR 84601.0500), 20g peptone (VWR 84620.0500), 20g dextrose (Fisher Life Sci G/0500/53). *Acetobacter pomorum* (DmCS004) was streaked and grown on M9+lactate plates (1.5% agar), before growing single colonies in liquid M9+lactate at 30°C with shaking (225 rpm) for 2 days. (DmCS004). *L. brevis* (DmCS003) was streaked on YPD (1.5% agar), then grown in liquid YPD for two days at 30C in static culture. Cultures were pelleted, washed, resuspended and diluted in sterile PBS to an $OD_{600}$ of 0.2. 200 µL of bacterial suspensions were added to T75 flasks containing freshly-prepared axenic eggs.

Newly emerged flies transferred to fresh media using sterile technique in a laminar flow hood, (preservatives as per development) for the first three days of adulthood before gentle anaesthesia on $CO_2$. Males were removed and females allocated to SYA containing preservatives in sterile glass *Drosophila* vials, using aseptic technique in a laminair flow hood with a sterile paint brush and sterile petri dish on ice. In experiments using RU486 (Mifepristone, Cayman Chemical, CAY10006317), 2ml $l^{-1}$ of 100mM RU486 stock solution in EtOH was incorporated into media at 200µM final concentration, and fed from day 3 adulthood onwards. 2ml EtOH was incorporated as vehicle control in RU-free controls.

For lifespan assays, mated females were allocated to sterile glassware in groups of 15, and transferred to fresh food every 2–3 days. At each transfer, deaths and censors were scored until all flies were dead. For axenic and gnotobiotic lifespans, fly tipping was performed in a sterile laminar flow hood with aseptic technique.

**Table 3.** ***Drosophila*** **stocks used in this study.**

| Genotype | Abbreviation | Source | | |
|---|---|---|---|---|
| | | BDSC | VDRC | Other |
| ***UAS* lines** | | | | |
| *UAS-TK-shRNAi* | | | v330743 | |
| *UAS-TK-RNAi* | | B25800 | | |
| *UAS-TK-RNAi* | | | v103662 | |
| *UAS-TkR99D-RNAi* | | | v44369 | |
| *UAS-TkR86C-RNAi* | | | v107090 | |
| *UAS-Reaper* | | | | Cathy Slack, U. Warwick |
| *UAS-AkhR-RNAi* | | | | Kenneth Halberg, U. Copenhagen |
| *UAS-MCD8::GFP* | | | | Julia Cordero, U. Glasgow |
| ***Gene Switch* drivers** | | | | |
| *Daughterless-GeneSwitch;* | *DaGS* | | | Cathy Slack, U. Warwick |
| *Dilp2-GeneSwitch* | *Dilp-GS* | | | Nathan Woodling, U. Glasgow |
| *S106-GeneSwitch* | *S106-GS* | | | Cathy Slack, U. Warwick |
| *Elav-GeneSwitch* | *Elav-GS* | | | Nathan Woodling, U. Glasgow |
| *Mex-GeneSwitch* | *Mex-GS* | | | Jean-René Martin, CNRS Université Paris-Saclay |
| ***GAL4* drivers** | | | | |
| *Voila-GAL4* | | | | Julia Cordero, U. Glasgow |
| *Akh-GAL4* | | | | Kenneth Halberg, U. Copenhagen |
| *TK-T2A-GAL4* | *TK-GAL4* | | | Kim Rewitz, U. Copenhagen |
| *Chat-Gal80* | | | | Julia Cordero, U. Glasgow |
| *R57C10-Gal80* | | | | Kim Rewitz, U. Copenhagen |
| **Other** | | | | |
| *FoxO* $^{\Delta}$ | | | | Cathy Slack, U. Warwick |
| *White Dahomey-20* | *wD*$^{-20}$ | | | Enric Ureña & Nazif Alic, UCL. |

## Feeding and egg laying assays

Feeding rate was measured by quantifying average proboscis extension rate (PER) over 3-4 hours. Vials containing 5 mated females each were arranged on a benchtop in a 25°C room the night before behavioural observations to allow acclimation. Experimenters were blinded to vial ID and experimental condition. The following morning, PER was recorded by iteratively counting the instantaneous number of flies feeding per vial for 3-4 hours. The average of these measurements per each given vial was taken as that vial's feeding rate. At the end of the experiment, flies were disposed, and vials frozen. The next day eggs were counted from the same frozen vials.

## TAG assay

Female flies were collected and divided into groups of 5, 3 days post eclosion. Each group was weighed on a Thermo microbalance to an accuracy of .01 mg. Weighed females were added to a sterile 2mL screw-cap tube containing sterile 1.4mm ceramic beads and 125 µL of TET buffer (10mM Tris, 1mM EDTA, pH 8.0, 0.1% (v/v) Triton-X-100). Samples were homogenised for 30s at max speed. After homogenisation, samples were incubated at 72°C for 15 min in a dry bath to inactivate lipases and other enzymes. Then samples were centrifuged at max speed for 5 min at 4°C. From each sample 3 µL of the supernatant was added to a clear, flat bottom 96 well plate. Standard curves were generated using a glycerol stock ranging from 1-0 ug/ul. Triglyceride levels were assayed by the method of Fossati and Prencipe(Fossati and Prencipe, 1982), with absorbance measured at 540 nm in a Thermo MultiSkan FC plate reader.

## RNA extraction

RNA was extracted from whole flies by the addition of 10 female flies per sample to sterile 2 mL screw cap tubes containing 600 µL of TRIzol (Ambion by Life technologies) and homogenised in a Ribolyzer and running at 6.5 (max) for 10 seconds. 200 µL chloroform was added, briefly vortexed, and incubated at RT for 3 min. Samples were centrifuged 12,000rpm for 15m at 4°C, 250µl of isopropanol was added to supernatants, vortexed, and incubated at RT for 10 minutes. Samples were then centrifuged at 12,000rpm for 10m at 4°C. The liquid was removed and discarded, while the pellet containing the RNA was washed with 1mL of 75% ethanol. Samples were centrifuged at 12,000 rpm for 10m at 4°C. The liquid was removed without disturbing the pellet, and the tubes were dried at RT with caps opened. Pellets were reconstituted in DEPC water (10µl/fly) and stored at −80°C.

## 16s amplicon sequencing

DNA was extracted from whole guts, dissected from 3 day-old wild-type stock females (i.e. conventionally-reared and bearing a complete microbiota, 5 per sample, 10 samples total) after 1 minute surface-sterilisation in 70% EtOH. Guts were deposited immediately into TRIzol and homogenised in a ribolyser. 300µl chloroform was added and inverted followed by 3 mins incubation at room temperature, 15 minutes centrifugation at 12000g. Aqueous layer was removed before 500µl back extraction buffer (4M Guanidine thiocyanate, 50 mM sodium citrate, 1M Tris base) was added and mixed by inverting. Samples were spun again at 12000g for 30 minutes and aqueous phase transferred to new tubes with 400 µl isopropanol. Samples were mixed by inversion, centrifuged 15 minutes at 12000g. Pellets were washed in 70% ethanol, dried, and resuspended in 50 µl TE buffer.

Library prep was performed by the University of Glasgow MVLS Shared Research Facility from 12.5ng input DNA per sample, with an initial PCR using Accustart PCRToughMix and 0.5µl 10mM primers against 16s V3/V4 region (FWD TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG, REV GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC), using 25 cycles at an annealing temperature of 60°C, followed by barcoding with Illumina Nextera XT 1 primers, cleanup with 0.9X Spri Select beads (Beckman Coulter), and washing in 80% EtOH. Libraries were

quantified by Qubit HS DNA assay and size distributions checked on an Agilent Bioanalyser. Pooled libraries were sequenced on an Illumina MiSeq in paired end mode (300bp) using a 600 cycle cartridge kit, 5% PhiX, to an average depth of 170K reads per sample.

## RT-qPCR

RNA samples were quantified by spectrophotometry using a Nanodrop 1000 UV-Vis spectrophotometer (ThermoScientific). First strand synthesis of cDNA was performed using the SuperScriptTM Reverse Transcriptase (RT) kit (Invitrogen), following the manufacturer's protocol (using 2µg of RNA). The PCR profile was as follows: 95°C for 2 minutes; 94°C denaturation for 30 seconds, 60°C annealing for 30 seconds, 72°C extension for 30 seconds each for 40 cycles, with a final extension at 72°C for 5 minutes. The amplicon was migrated through a 1% (w/v) agarose gel. To test for relative gene expression levels, RT-qPCR was performed. Each well contained 2 µl cDNA, 1.5 µl 10 mM forward primer, 1.5 µl 10 mM reverse primer, 3 µl water, 10 µl SYBR green PCR master mix, and 2 ul ROX (Qiagen, UK). The following PCR profile was used: 95°C for 5 minutes; 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds each for 40 cycles, and a final extension at 72°C for 5 minutes. Mean cycle threshold (Ct) values for duplicates of each of the studied genes were derived by subtracting the combined mean Ct value of constitutively expressed housekeeping genes *alcohol dehydrogenase* (*ADH*) or *tubulin*. Primers shown in Table 4.

## Antibody staining and imaging

Whole guts were extracted from 10-day old female flies (axenic, gnotobiotic, and axenic on 1µM TSA), and fixed in 4% paraformaldehyde for 30m in a 12 well plate. Guts were then incubated in 1 mL of PBST (1X PBS with 0.1% triton X-100) overnight with slight shaking at 4°C. PBST was carefully aspirated from sample wells, and guts were then washed with 1 mL of Blocking buffer (10% 1X PBS, 1% triton X-100, 2.5% Normal Goat Serum) at 4°C overnight. Blocking buffer was removed, and 0.5 mL of acetyl-lysine antibody (Cell Signaling Technologies 9441) diluted 1/800 in blocking buffer was added to the wells, and incubated overnight at 4°C with slight shaking. Guts were then washed 3X with PBST for 10 minutes, followed by a 1 hr RT PBST wash with slight shaking. 0.5 mL of secondary antibody secondary antibodies diluted 1/200 in PBST was added to the guts, and incubated overnight at 4°C. Guts were washed 3X in PBST for 10 minutes. Guts were then mounted in Vectashield for imaging.

Corpora cardiaca were dissected, fixed and washed as above, stained with anti-Akh (gift from Kenneth Halberg) diluted 1:1000, and stained with secondary antibodies as above.

## Confocal microscopy

Imaging was performed on a Zeiss LSM 880 confocal microscope and processed using Zeiss Zen Blue (v3.8) and NIH ImageJ software 1.53a prior to transfer to Adobe Photoshop and Illustrator (Creative Cloud 2025; CA, USA) for final presentation.

## Data analysis

All enumerated data were plotted and analysed in R 4.4.2. (Team, 2024). Kaplan-Meier curves were plotted with `survminer::ggsurvplot_facet`. Other quantitative data were plotted with ggplot2. Survival data were analysed with CoxPH models (`survival::coxph`). Fecundity data were analysed with negative binomial models (`lme4::glmer.nb`). All other data were analysed with `stats::lm` (after logit transformation for proportion flies feeding). Post-hoc tests were performed with `emmeans::joint_tests` and `emmeans::pairs`, coefficients were extracted from `emmeans::emmip`. Survival indices were generated by applying `emmeans::emmip` to coxph models, multiplied by −1, such that higher values corresponded to longer lifespan.

Published transcriptome data (Bost et al., 2018) were reanalysed by FastQC and quantified with Salmon. The previous publication by Bost et al(Bost et al., 2018) presented bulk RNAseq on midguts of flies reared under axenic conditions or with a 5-species gnotobiotic microbiota comprising *Acetobacter* and *Levilactobacillus* species. The original analysis presented excluded

| Target | Forward | Reverse |
|---|---|---|
| *TK* | TTCATATGCGCCCTCTGAGCG | ATGAAGCTGGACGTGGGCGC |
| *Bursicon* | CGCCACGAGAACAACAAGGT | CATCCAGGATACTGGAGCAC |
| *ADH* | GCTAACGAGTACTTGCATCTCTTC | AGACCGGCAACGAAAATCAC |
| *Tubulin* | TCGCCGTCACCGGAGTCCAT | TGGGCCCGTCTGGACCACAA |
| *Dilp2* | ATGGTGTGCGAGGAGTATAATCC | TCGGCACCGGGCATG |
| *Dilp3* | AGAGAACTTTGGACCCCGTGAA | TGAACCGAACTATCACTCAACAGTCT |
| *Dilp5* | GAGGCACCTTGGGCCTATTC | CATGTGGTGAGATTCGGAGCTA |

**Table 4.** **Primers for RT-qPCR**

expression of ~70% of genes in the fly genome, and hormones were largely excluded. We reanalysed the raw data using Salmon (Patro et al., 2017) to quantify expression, which quantified expression of 10,290 genes, including 14 of the 15 enteroendocrine hormone genes (except *bursicon*). We tested for differential expression using DESeq2, identifying 470 differentially expressed genes (FDR ≤ 0.01, ≥2-fold difference in mean expression). These 470 genes included four peptide hormones: *Tachykinin (Tk), Allostatin C (AstC), Ion Transport Peptide (ITP), and Neuropeptide F (NPF)*. Since *Burs* was not quantified by the Salmon algorithm, we tested independently whether its expression was modulated by microbiota by extracting RNA from midguts of CR and axenic flies.

## Supplementary figures

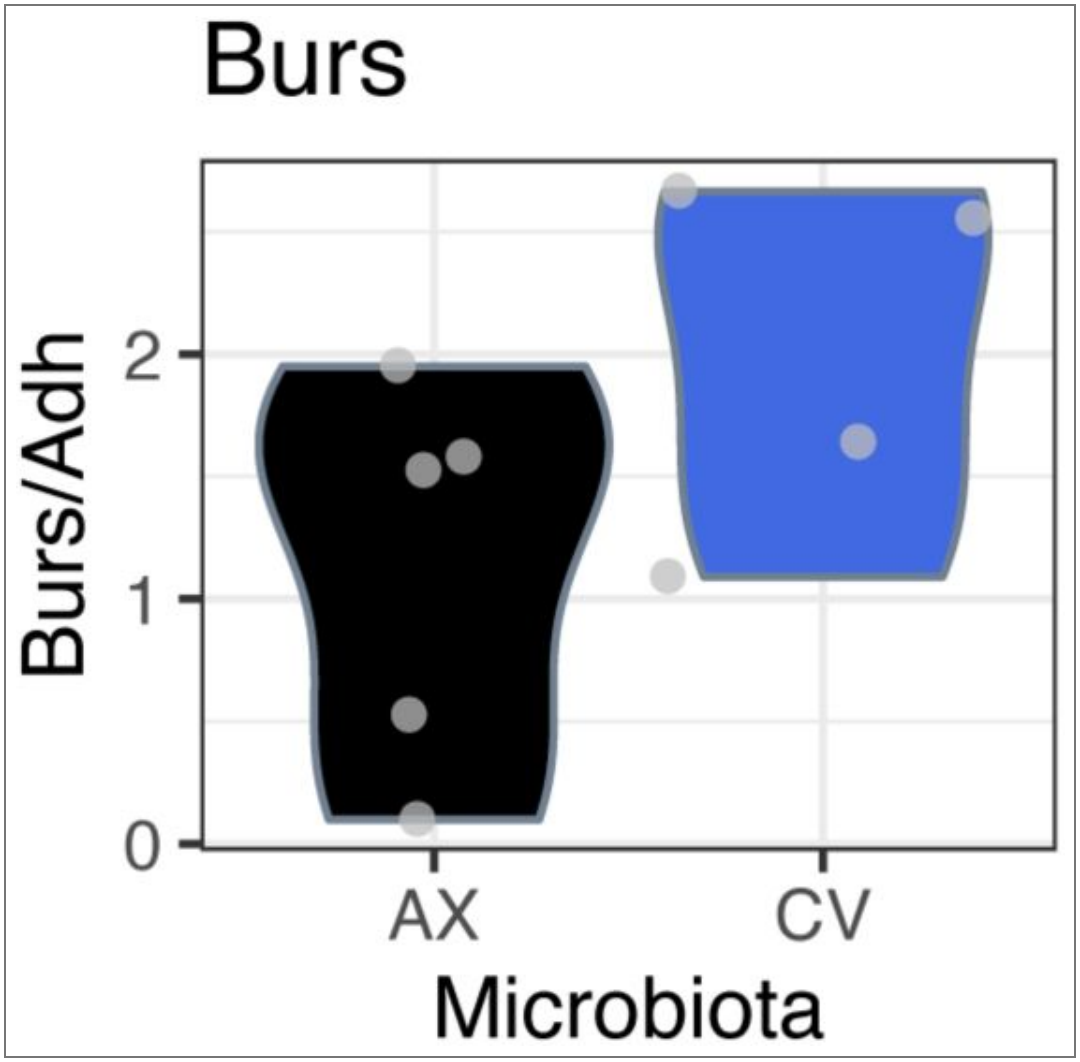



**Figure S1. Microbiota do not alter intestinal *Burs* expression.** Expression was quantified by RT-qPCR (using cDNA generated from midgut to complement RNA-seq data) because transcripts were not quantified from RNA-seq data (Figure 1). No significant difference in expression was detected (t=-1.65, p=0.15). AX=axenic, CV=conventionally-reared. Expression was quantified relative to *Adh*, as a housekeeping gene that RNAseq data showed to not be influenced by microbiota (Figure S2)

**Figure S2. Microbiota do not alter intestinal *Adh* expression.**

Expression was quantified from public RNAseq data (Bost et al., 2018). Statistics show log2 fold-change and p-value (unadjusted) from DESeq2 analysis of reads quantified with Salmon.

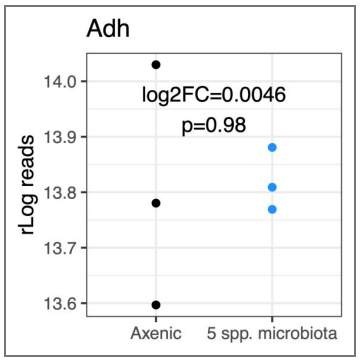



**Figure S3. Lifespan extension in conventional flies by induction of an independent $Tk^{RNAi}$.**

To validate result show in Figure 2A a distinct construct (Vienna *Drosophila* Stock Center #25800) was expressed under control of *DaGS.* Log-rank test p<0.01. -RU condition 108 deaths, 43 censors; +RU condition 124 deaths, 26 censors.

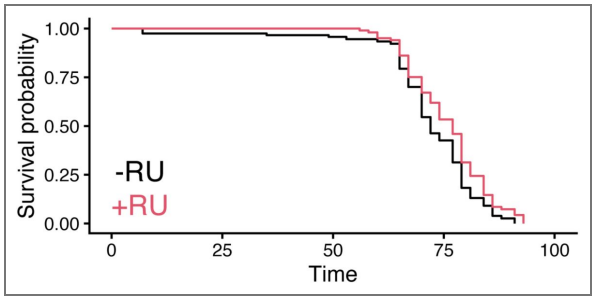



**Figure S4. Lifespan extension by feeding RU to CR *DaGS;UAS-*$Tk^{RNAi}$ flies is not attributable to off-target effects of RU or GeneSwitch activation.**

Kaplan-Meier plot shows impact of feeding RU to *DaGS/+* flies reared with complete microbiota. No effect of RU was detected (Cox Proportional Hazards p=0.5).

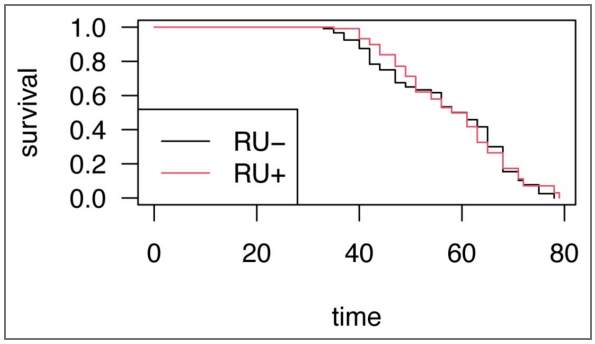


**Figure S5. CFU. *TkRNAi* does not affect bacterial load in CR flies.**

Violin plots show CFUs of conventionally-reared female adults of the indicated genotypes (all with *DaGS*), after one week of feeding on RU or vehicle control. All conditions n=10. No effects were detected of presence/absence of *UAS-Tk-RNAi* (ANOVA of log CFU counts, p=0.32), RU (p=0.23), nor genotype:RU interaction (p=0.25).

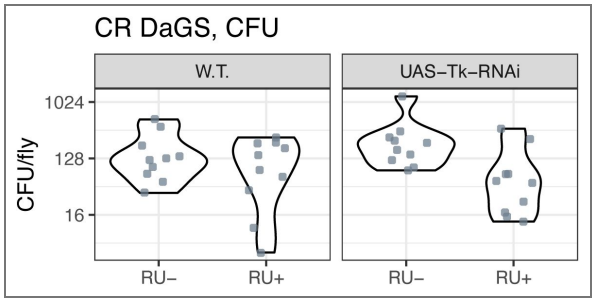



**Figure S6. Interactive effect of *TkRNAi* and microbiota on TAG levels confirmed with independent RNAi construct.**

The experiment presented in Figure 2E (which used construct V330743) was repeated with an independent construct (V103668). The same result was replicated. Violin plots show density of datapoints, mean±CI95 given in white. ANOVA (Type 3 tests), microbiota:RU $F_{1,26}$=37.86, p=1.66e-06. Statistics on brackets are from *post-hoc* EMM analyses of linear model, when significant differences were detected.

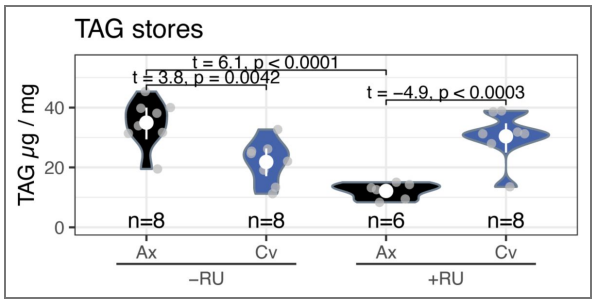



**Figure S7. Modulation of *Tk* expression by microbiota correlates lifespan outcome.**

**A.** RT-qPCR indicates elevated *Tk* expression in *Ap*-flies above levels in axenics and *Lb*-flies, and above levels in all conditions expressing $Tk^{RNAi}$. Expression in *Lb*-flies was significantly greater than in conditions expressing $Tk^{RNAi}$, but not significantly greater than axenics without $Tk^{RNAi}$ expression. Statistics on brackets are from *post-hoc* EMM analyses of linear model, when significant differences were detected. **B.** *Tk* expression differences across microbiota and $Tk^{RNAi}$ conditions correlate lifespan differences. *Tk* expression from panel A is plotting against survival coefficients of lifespan experiment shown in Figure 3c-d.

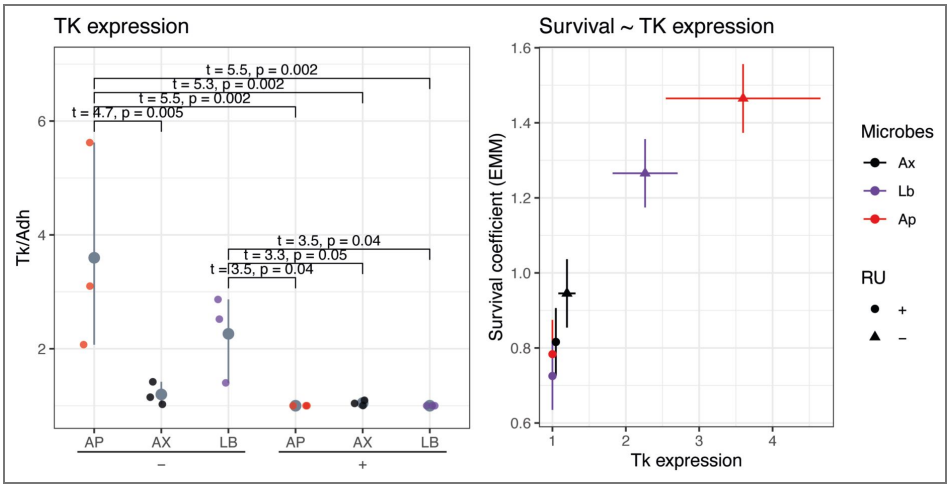


**Figure S8. Intersectional Gal80 strategy to direct Gal4 activity to enteroendocrine cells, sparing CNS.**

As per Medina et al (Medina et al., 2024), we tested the capacity of Gal80 expressed under control of the *ChAT* promoter to repress Gal4 expressed under control of the *Voila* promoter, assayed by expression of *UAS-mCD8::GFP*. **A.** Representative confocal micrographs of CNS, and **B.** Quantification of Voila>GFP intensity in brain and CNS, in presence/absence of *Voila Gal80*. **C.** Representative confocal micrographs of midgut (region 4). **D.** Number of GFP+ cells/ROI in presence/absence of *Voila Gal80*.

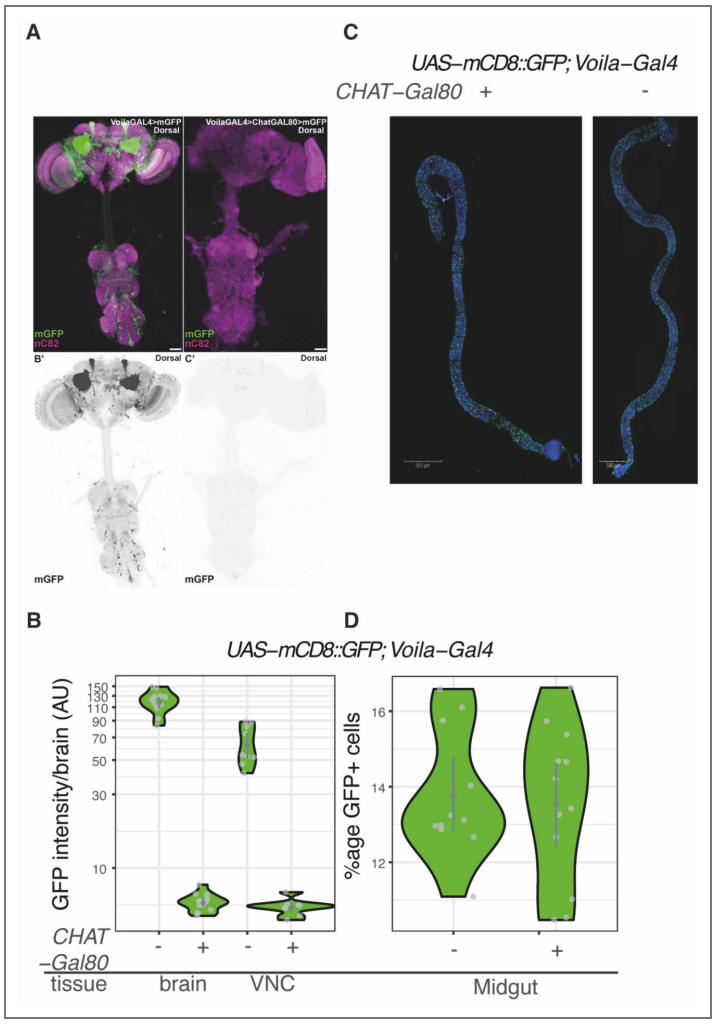
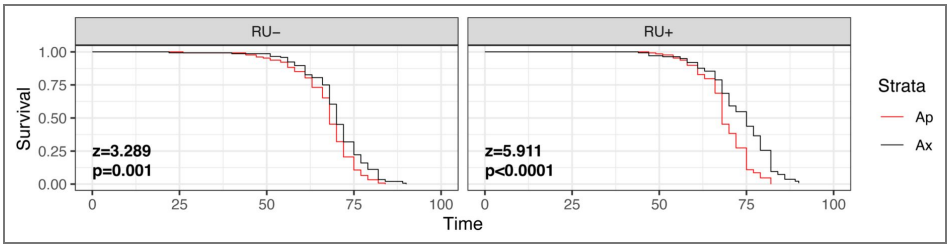



**Figure S9. *TkR99D*$^{RNAi}$ induction in enterocytes does not block impact of *A. pomorum* on lifespan.**

Expression was driven with *Mex1-GeneSwitch*. Statistics in panel from Cox proportional hazards model, reporting post-hoc (EMM) analysis for effect of RU per microbiota condition.



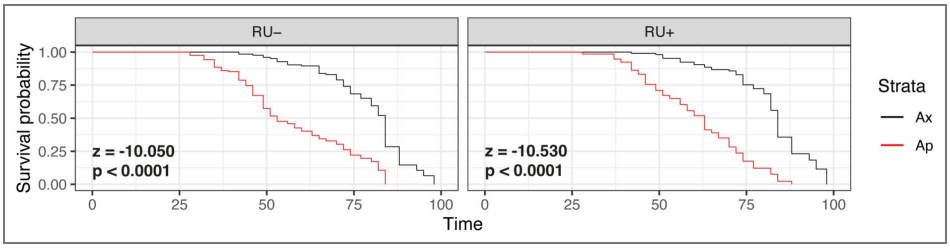



**Figure S10. *AkhR*$^{RNAi}$ induction in fat body (and gut) does not block impact of *A. pomorum* on lifespan.**

Expression was driven with *S106-GeneSwitch*. Statistics in panel from Cox proportional hazards model, reporting post-hoc (EMM) analysis for effect of RU per microbiota condition.

## Acknowledgements

We thank Julia Cordero for helpful discussion, reagents and training; Nathan Woodling and Colin Selman for comments on the manuscript; and Kim Rewitz, Kenneth Halberg, Jean-René Martin and Peter Newell for reagents. The authors gratefully acknowledge the Cellular Analysis Facility at the University of Glasgow – particularly David McGuinness – for support and assistance in this work. This work was funded by a UKRI Future Leaders Fellowship (MR/S033939/1 and MR/Y019660/1, AD), BBSRC (BB/W510658/1, DRS and AD), a University of Glasgow Lord Kelvin Adam Smith Fellowship (AD), a University of Glasgow Lord Kelvin Adam Smith Scholarship (RI), and funds from the University of Glasgow Wellcome Trust Institutional Strategic Support award (RI and AD).

## Additional information

### Funding

| Funder | Grant reference number | Author |
|---|---|---|
| UKRI \| Medical Research Council (MRC) | MR/S033939/1 | Diana Marcu |
| UKRI \| Medical Research Council (MRC) | MR/Y019660/1 | Diana Marcu |
| University of Glasgow | | Diana Marcu |
| UKRI \| Biotechnology and Biological Sciences Research Council (BBSRC) | BB/W510658/1 | David Sannino |

## References

**Ahrentløv N,** Kubrak O, Lassen M, Malita A, Koyama T, Frederiksen AS, Sigvardsen CM, John A, Madsen PEH, Halberg KV, *et al.* (2025) Protein-responsive gut hormone tachykinin directs food choice and impacts lifespan. *Nat Metab* **7**:1223-1245 https://doi.org/10.1038/s42255-025-01267-0

**Alic N,** Tullet JM, Niccoli T, Broughton S, Hoddinott MP, Slack C, Gems D, Partridge L (2014) Cell-nonautonomous effects of dFOXO/DAF-16 in aging. *Cell reports* **6** https://doi.org/10.1016/j.celrep.2014.01.015

**Bass TM,** Grandison RC, Wong R, Martinez P, Partridge L, Piper MDW (2007) Optimization of Dietary Restriction Protocols in Drosophila. *Journals Gerontology Ser* **62**:1071-1081 https://doi.org/10.1093/gerona/62.10.1071

**Birse RT,** Söderberg JA, Luo J, Winther ÅM, Nässel DR (2011) Regulation of insulin-producing cells in the adult Drosophila brain via the tachykinin peptide receptor DTKR. *The Journal of Experimental Biology* **214**:4201-4208 https://doi.org/10.1242/jeb.062091

**Bjedov I,** Toivonen J, Kerr F, Slack C, Jacobson J. (2010) Mechanisms of life span extension by rapamycin in the fruit fly Drosophila melanogaster. *Cell Metab* **11**:35-46

**Boehme M,** Guzzetta KE, Wasén C, Cox LM (2022) The gut microbiota is an emerging target for improving brain health during ageing. *Gut Microbiome* **4**:e2 https://doi.org/10.1017/gmb.2022.11

**Bost A,** Franzenburg S, Adair KL, Martinson VG, Loeb G, Douglas AE (2018) How gut transcriptional function of Drosophila melanogaster varies with the presence and composition of the gut microbiota. *Mol Ecol* **27**:1848-1859 https://doi.org/10.1111/mec.14413

**Broderick NA,** Buchon N, Lemaitre B (2014) Microbiota-induced changes in drosophila melanogaster host gene expression and gut morphology. *mBio* **5**:e01117-14 https://doi.org/10.1128/mbio.01117-14

**Broughton SJ,** Piper MD, Ikeya T, Bass TM, Jacobson J, Driege Y, Martinez P, Hafen E, Withers DJ, Leevers SJ, *et al.* (2005) Longer lifespan, altered metabolism, and stress resistance in Drosophila from ablation of cells making insulin-like ligands. *Proceedings of the National Academy of Sciences of the United States of America* **102**:3105-3110 https://doi.org/10.1073/pnas.0405775102

**Brown JJ,** Jandová A, Jeffs CT, Higgie M, Nováková E, Lewis OT, Hrček J (2023) Microbiome structure of a wild Drosophila community along tropical elevational gradients and comparison to laboratory lines. *bioRxiv* 2021.07.28.454263 https://doi.org/10.1101/2021.07.28.454263

**Brummel T,** Ching A, Seroude L, Simon AF, Benzer S (2004) Drosophila lifespan enhancement by exogenous bacteria. *Proceedings of the National Academy of Sciences of the United States of America* **101**:12974-12979 https://doi.org/10.1073/pnas.0405207101

**Buchon N,** Broderick NA, Chakrabarti S, Lemaitre B (2009) Invasive and indigenous microbiota impact intestinal stem cell activity through multiple pathways in Drosophila. *Genes & development* **23**:2333-44 https://doi.org/10.1101/gad.1827009

**Cabreiro F,** Au C, Leung K-Y, Vergara-Irigaray N, Cochemé HM, Noori T, Weinkove D, Schuster E, Greene N, Gems D (2013) Metformin Retards Aging in C. elegans by Altering Microbial Folate and Methionine Metabolism. *Cell* **153**:228-239 https://doi.org/10.1016/j.cell.2013.02.035

**Chandler JA,** Lang JM, Bhatnagar S, Eisen JA, Kopp A (2011) Bacterial Communities of Diverse Drosophila Species: Ecological Context of a Host–Microbe Model System. *PLoS Genet* **7**:e1002272 https://doi.org/10.1371/journal.pgen.1002272

**Chatzispyrou IA,** Held NM, Mouchiroud L, Auwerx J, Houtkooper RH (2015) Tetracycline Antibiotics Impair Mitochondrial Function and Its Experimental Use Confounds Research. *Cancer Res* **75**:4446-4449 https://doi.org/10.1158/0008-5472.can-15-1626

**Clancy DJ,** Gems D, Harshman LG, Oldham S, Stocker H, Hafen E, Leevers SJ, Partridge L (2001) Extension of Life-Span by Loss of CHICO, a Drosophila Insulin Receptor Substrate Protein. *Science* **292**:104-106 https://doi.org/10.1126/science.1057991

**Clark RI,** Salazar A, Yamada R, Fitz-Gibbon S, Morselli M, Alcaraz J, Rana A, Rera M, Pellegrini M, Ja WW, *et al.* (2015) Distinct Shifts in Microbiota Composition during Drosophila Aging Impair Intestinal Function and Drive Mortality. *Cell reports* **12**:1656-67 https://doi.org/10.1016/j.celrep.2015.08.004

**Clark RI,** Walker DW (2017) Role of gut microbiota in aging-related health decline: insights from invertebrate models. *Cellular and Molecular Life Sciences* https://doi.org/10.1007/s00018-017-2671-1

**Cui R,** Medeiros T, Willemsen D, Iasi LNM, Collier GE, Graef M, Reichard M, Valenzano DR (2019) Relaxed Selection Limits Lifespan by Increasing Mutation Load. *Cell* https://doi.org/10.1016/j.cell.2019.06.004

**Dinan TG,** Cryan JF (2017) Gut instincts: microbiota as a key regulator of brain development, ageing and neurodegeneration. *J Physiol* **595**:489-503 https://doi.org/10.1113/jp273106

**Dobson AJ,** Chaston JM, Douglas AE (2016) The Drosophila transcriptional network is structured by microbiota. *BMC genomics* **17**:975

**Dobson AJ,** Chaston JM, Newell PD, Donahue L, Hermann SL, Sannino DR, Westmiller S, Wong AC, Clark AG, Lazzaro BP, *et al.* (2015) Host genetic determinants of microbiota-dependent nutrition revealed by genome-wide analysis of Drosophila melanogaster. *Nature communications* **6**:6312 https://doi.org/10.1038/ncomms7312

**Flatt T** (2009) Ageing: Diet and longevity in the balance. *Nature* **462**:989-990 https://doi.org/10.1038/462989a

**Fossati P,** Prencipe L (1982) Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. *Clin Chem* **28**:2077-2080 https://doi.org/10.1093/clinchem/28.10.2077

**Gäde G,** Marco HG. (2022) The Adipokinetic Peptides of Hemiptera: Structure, Function, and Evolutionary Trends. *Front Insect Sci* **2**:891615 https://doi.org/10.3389/finsc.2022.891615

**Gagnon E,** Mitchell PL, Manikpurage HD, Abner E, Taba N, Esko T, Ghodsian N, Thériault S, Mathieu P, Arsenault BJ (2023) Impact of the gut microbiota and associated metabolites on cardiometabolic traits, chronic diseases and human longevity: a Mendelian randomization study. *J Transl Med* **21**:60 https://doi.org/10.1186/s12967-022-03799-5

**Good T,** Tatar M (2001) Age-specific mortality and reproduction respond to adult dietary restriction in Drosophila melanogaster. *J Insect Physiol* **47**:1467-1473

**Gordon HA,** Bruckner-Kardoss E, Wostmann BS (1966) Aging in germ-free mice: life tables and lesions observed at natural death. *Journal of gerontology* **21**:380-7

**Grandison RC,** Piper MD, Partridge L (2009) Amino-acid imbalance explains extension of lifespan by dietary restriction in Drosophila. *Nature* **462**:1061-1064 https://doi.org/10.1038/nature08619

**Guo X,** Yin C, Yang F, Zhang Y, Huang H, Wang J, Deng B, Cai T, Rao Y, Xi R (2019) The Cellular Diversity and Transcription Factor Code of Drosophila Enteroendocrine Cells. *Cell Reports* **29**:4172-4185.e5. https://doi.org/10.1016/j.celrep.2019.11.048

**Harrison DE,** Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS, *et al.* (2009) Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature* **460**:392-395 https://doi.org/10.1038/nature08221

**Houthoofd K,** Braeckman BP, Lenaerts I, Brys K, Vreese AD, Eygen SV, Vanfleteren JR (2002) Axenic growth up-regulates mass-specific metabolic rate, stress resistance, and extends life span in Caenorhabditis elegans. *Experimental gerontology* **37**:1371-8

**Huang J-H,** Douglas AE (2015) Consumption of dietary sugar by gut bacteria determines Drosophila lipid content. *Biology Letters* **11**:20150469 https://doi.org/10.1098/rsbl.2015.0469

**Hung R-JJ,** Hu Y, Kirchner R, Liu Y, Xu C, Comjean A, Tattikota SG, Li F, Song W, Sui SH, *et al.* (2020) A cell atlas of the adult Drosophila midgut. *Proceedings of the National Academy of Sciences of the United States of America* https://doi.org/10.1073/pnas.1916820117

**Johansen J,** Atarashi K, Arai Y, Hirose N, Sørensen SJ, Vatanen T, Knip M, Honda K, Xavier RJ, Rasmussen S, *et al.* (2023) Centenarians have a diverse gut virome with the potential to modulate metabolism and promote healthy lifespan. *Nat Microbiol* **8**:1064-1078 https://doi.org/10.1038/s41564-023-01370-6

**Jugder B-E,** Kamareddine L, Watnick PI (2021) Microbiota-derived acetate activates intestinal innate immunity via the Tip60 histone acetyltransferase complex. *Immunity* **54**:1683-1697.e3. https://doi.org/10.1016/j.immuni.2021.05.017

**Kenyon C,** Chang J, Gensch E, Rudner A, Tabtiang R (1993) A C. elegans mutant that lives twice as long as wild type. *Nature* **366**:461 https://doi.org/10.1038/366461a0

**Kim B,** Kanai MI, Oh Y, Kyung M, Kim E-K, Jang I-H, Lee J-H, Kim S-G, Suh GSB, Lee W-J (2021) Response of the microbiome–gut–brain axis in Drosophila to amino acid deficit. *Nature* **593**:570-574 https://doi.org/10.1038/s41586-021-03522-2

**Kubrak O,** Koyama T, Ahrentløv N, Jensen L, Malita A, Naseem MT, Lassen M, Nagy S, Texada MJ, Halberg KV, *et al.* (2022) The gut hormone Allatostatin C/Somatostatin regulates food intake and metabolic homeostasis under nutrient stress. *Nat Commun* **13**:692 https://doi.org/10.1038/s41467-022-28268-x

**Leader DP,** Krause SA, Pandit A, Davies SA, Dow JAT (2018) FlyAtlas 2: a new version of the Drosophila melanogaster expression atlas with RNA-Seq, miRNA-Seq and sex-specific data. *Nucleic Acids Res* **46**:D809-D815 https://doi.org/10.1093/nar/gkx976

**Lin H-H,** Kuang MC, Hossain I, Xuan Y, Beebe L, Shepherd AK, Rolandi M, Wang JW (2022) A nutrient-specific gut hormone arbitrates between courtship and feeding. *Nature* **602**:632-638 https://doi.org/10.1038/s41586-022-04408-7

**Liu X,** Nagy P, Bonfini A, Houtz P, Bing X-L, Yang X, Buchon N (2022) Microbes affect gut epithelial cell composition through immune-dependent regulation of intestinal stem cell differentiation. *Cell Reports* **38**:110572 https://doi.org/10.1016/j.celrep.2022.110572

**Liu X,** Zou L, Nie C, Qin Y, Tong X, Wang J, Yang H, Xu X, Jin X, Xiao L, *et al.* (2023) Mendelian randomization analyses reveal causal relationships between the human microbiome and longevity. *Sci Rep* **13**:5127 https://doi.org/10.1038/s41598-023-31115-8

**Mair W,** Piper MD, Partridge L (2005) Calories Do Not Explain Extension of Life Span by Dietary Restriction in Drosophila. *PLoS Biology* **3**:e223 https://doi.org/10.1371/journal.pbio.0030223

**Malita A,** Kubrak O, Koyama T, Ahrentløv N, Texada MJ, Nagy S, Halberg KV, Rewitz K (2022) A gut-derived hormone suppresses sugar appetite and regulates food choice in Drosophila. *Nat Metab* **4**:1532-1550 https://doi.org/10.1038/s42255-022-00672-z

**Matthews MK,** Wilcox H, Hughes R, Veloz M, Hammer A, Banks B, Walters A, Schneider KJ, Sexton CE, Chaston JM (2020) Genetic Influences of the Microbiota on the Life Span of Drosophila melanogaster. *Appl Environ Microb* **86** https://doi.org/10.1128/aem.00305-20

**Medina AB,** Perochon J, Johnson C, Polcowñuk S, Tian Y, Yu Y, Cordero JB (2024) Neuroendocrine Control of Intestinal Regeneration Through the Vascular Niche in Drosophila. *bioRxiv* 2024.09.10.612352 https://doi.org/10.1101/2024.09.10.612352

**Miller RA,** Harrison DE, Astle CM, Fernandez E, Flurkey K, Han M, Javors MA, Li X, Nadon NL, Nelson JF, *et al.* (2014) Rapamycin-mediated lifespan increase in mice is dose and sex dependent and metabolically distinct from dietary restriction. *Aging Cell* **13**:468-477 https://doi.org/10.1111/acel.12194

**Moullan N,** Mouchiroud L, Wang X, Ryu D, Williams EG, Mottis A, Jovaisaite V, Frochaux MV, Quiros PM, Deplancke B, *et al.* (2015) Tetracyclines Disturb Mitochondrial Function across Eukaryotic Models: A Call for Caution in Biomedical Research. *Cell Rep* **10**:1681-1691 https://doi.org/10.1016/j.celrep.2015.02.034

**Musselman LP,** Kühnlein RP, Suarez RK, Hoppeler HH (2018) Drosophila as a model to study obesity and metabolic disease. *J Exp Biol* **221**:jeb163881 https://doi.org/10.1242/jeb.163881

**Nässel DR** (2025) What Drosophila can tell us about state-dependent peptidergic signaling in insects. *Insect Biochem Mol Biol* 104275 https://doi.org/10.1016/j.ibmb.2025.104275

**Nässel DR,** Zandawala M, Kawada T, Satake H (2019) Tachykinins: Neuropeptides That Are Ancient, Diverse, Widespread and Functionally Pleiotropic. *Front Neurosci* **13**:1262 https://doi.org/10.3389/fnins.2019.01262

**Newell PD,** Chaston JM, Wang Y, Winans NJ, Sannino DR, Wong ACN, Dobson AJ, Kagle J, Douglas AE (2014) In vivo function and comparative genomic analyses of the Drosophila gut microbiota identify candidate symbiosis factors. *Front Microbiol* **5**:576 https://doi.org/10.3389/fmicb.2014.00576

**Newell PD,** Douglas AE (2014) Interspecies Interactions Determine the Impact of the Gut Microbiota on Nutrient Allocation in Drosophila melanogaster. *Appl Environ Microbiol* **80**:788-796 https://doi.org/10.1128/aem.02742-13

**Obata F,** Fons CO, Gould AP (2018) Early-life exposure to low-dose oxidants can increase longevity via microbiome remodelling in Drosophila. *Nat Commun* **9**:975 https://doi.org/10.1038/s41467-018-03070-w

**OToole P,** Jeffery I. (2015) Gut microbiota and aging. *Science* **350**:1214-1215 https://doi.org/10.1126/science.aac8469

**Partridge L,** Gems D, Withers DJ (2005) Sex and Death: What Is the Connection?. *Cell* **120** https://doi.org/10.1016/j.cell.2005.01.026

**Patro R,** Duggal G, Love MI, Irizarry RA, Kingsford C (2017) Salmon provides fast and bias-aware quantification of transcript expression. *Nat Methods* **14**:417-419 https://doi.org/10.1038/nmeth.4197

**Pryor R,** Norvaisas P, Marinos G, Best L, Thingholm LB, Quintaneiro LM, Haes WD, Esser D, Waschina S, Lujan C, *et al.* (2019) Host-Microbe-Drug-Nutrient Screen Identifies Bacterial Effectors of Metformin Therapy. *Cell* https://doi.org/10.1016/j.cell.2019.08.003

**Qansuwa EM,** Atalah HN, Abdelkader MS, Russell AE, Dakhlallah D, Brown CM (2024) The Contribution of the Gut-Brain-Microbiota Axis to Brain Health Throughout the Lifespan. In: Essa MM (Ed). *Handbook of Neurodegenerative Disorders* Singapore: Springer Singapore. pp. 17-41 https://doi.org/10.1007/978-981-99-7557-0_2

**Regan JC,** Khericha M, Dobson AJ, Bolukbasi E, Rattanavirotkul N, Partridge L (2016) Sex difference in pathology of the ageing gut mediates the greater response of female lifespan to dietary restriction. *eLife* **5**:e10956 https://doi.org/10.7554/elife.10956

**Regan JC,** Partridge L (2013) Gender and longevity: Why do men die earlier than women? Comparative and experimental evidence. *Best Pr Res Clin Endocrinol Metab* **27**:467-479 https://doi.org/10.1016/j.beem.2013.05.016

**Rera M,** Clark R, Walker D (2012) Intestinal barrier dysfunction links metabolic and inflammatory markers of aging to death in Drosophila. *Proceedings of the National Academy of Sciences* **109** https://doi.org/10.1073/pnas.1215849110

**Rewitz K,** Ahrentlov N, Kubrak O, Malita A, Koyama T, John A, Madsen P, Halberg K, Nagy S, Texada M (2024) Protein-responsive gut hormone Tachykinin directs food choice and impacts lifespan in Drosophila. *Research Square* https://doi.org/10.21203/rs.3.rs-3837414/v1

**Ronayne CT,** Jackson TD, Bennett CF, Perry EA, Kantorovic N, Puigserver P (2023) Tetracyclines activate mitoribosome quality control and reduce ER stress to promote cell survival. *EMBO Rep* **24**:e57228 https://doi.org/10.15252/embr.202357228

**Sannino DR,** Dobson AJ (2023) Acetobacter pomorum in the Drosophila gut microbiota buffers against host metabolic impacts of dietary preservative formula and batch variation in dietary yeast. *Appl Environ Microbiol* **89**:e00165-23 https://doi.org/10.1128/aem.00165-23

**Sannino DR,** Dobson AJ, Edwards K, Angert ER, Buchon N (2018) The Drosophila melanogaster Gut Microbiota Provisions Thiamine to Its Host. *mBio* **9** https://doi.org/10.1128/mbio.00155-18

**Sass F,** Ma T, Ekberg JH, Kirigiti M, Ureña MG, Dollet L, Brown JM, Basse AL, Yacawych WT, Burm HB, *et al.* (2024) NK2R control of energy expenditure and feeding to treat metabolic diseases. *Nature* **635**:987-1000 https://doi.org/10.1038/s41586-024-08207-0

**Schwarzer M,** Makki K, Storelli G, Machuca-Gayet I, Srutkova D, Hermanova P, Martino ME, Balmand S, Hudcovic T, Heddi A, *et al.* (2016) Lactobacillus plantarum strain maintains growth of infant mice during chronic undernutrition. *Science (New York, NY)* **351**:854-7 https://doi.org/10.1126/science.aad8588

**Scopelliti A,** Cordero JB, Diao F, Strathdee K, White BH, Sansom OJ, Vidal M (2014) Local Control of Intestinal Stem Cell Homeostasis by Enteroendocrine Cells in the Adult Drosophila Midgut. *Current Biology* **24** https://doi.org/10.1016/j.cub.2014.04.007

**Seidel J,** Valenzano D (2018) The role of the gut microbiome during host ageing. *F1000Research* **7**:1086 https://doi.org/10.12688/f1000research.15121.1

**Selman C,** Lingard S, Choudhury AI, Batterham RL, Claret M, Clements M, Ramadani F, Okkenhaug K, Schuster E, Blanc E, *et al.* (2008) Evidence for lifespan extension and delayed age–related biomarkers in insulin receptor substrate 1 null mice. *Faseb J* **22**:807-818 https://doi.org/10.1096/fj.07-9261com

**Shin S,** Kim S-H, You H, Kim B, Kim AC, Lee K-A, Yoon J-H, Ryu J-H, Lee W-J (2011) *Drosophila* Microbiome Modulates Host Developmental and Metabolic Homeostasis via Insulin Signaling. *Science* **334**:670-674 https://doi.org/10.1126/science.1212782

**Shin SC,** Kim S-H, You H, Kim B, Kim AC, Lee K-A, Yoon J-H, Ryu J-H, Lee W-J (2011) *Drosophila* Microbiome Modulates Host Developmental and Metabolic Homeostasis via Insulin Signaling. *Science* **334**:670-674 https://doi.org/10.1126/science.1212782

**Slack C,** Alic N, Foley A, Cabecinha M, Hoddinott MP, Partridge L (2015) The Ras-Erk-ETS-Signaling Pathway Is a Drug Target for Longevity. *Cell* **162**:72-83 https://doi.org/10.1016/j.cell.2015.06.023

**Slack C,** Giannakou ME, Foley A, Goss M, Partridge L (2011) dFOXO-independent effects of reduced insulin-like signaling in Drosophila. *Aging Cell* **10**:735-748 https://doi.org/10.1111/j.1474-9726.2011.00707.x

**Slagboom PE,** Berg N van den, Deelen J. (2017) Phenome and genome based studies into human ageing and longevity: An overview. *Biochimica Et Biophysica Acta Mol Basis Dis* **1864**:2742-2751 https://doi.org/10.1016/j.bbadis.2017.09.017

**Smith P,** Willemsen D, Popkes M, Metge F, Gandiwa E, Reichard M, Valenzano DR (2017) Regulation of life span by the gut microbiota in the short-lived African turquoise killifish. *eLife* **6** https://doi.org/10.7554/elife.27014

**Snyder D,** Pollard M, Wostmann B, Luckert P (1990) Life span, morphology, and pathology of diet-restricted germ-free and conventional Lobund-Wistar rats. *Journal of gerontology* **45**:B52-8 https://doi.org/10.1093/geronj/45.2.b52

**Song W,** Veenstra JA, Perrimon N (2014) Control of Lipid Metabolism by Tachykinin in Drosophila. *Cell Reports* https://doi.org/10.1016/j.celrep.2014.08.060

**Staubach F,** Baines JF, Kuenzel S, Bik EM, Petrov DA. (2012) Host species and environmental effects on bacterial communities associated with Drosophila in the laboratory and in the natural environment. *arXiv* https://doi.org/10.48550/arxiv.1211.3367

**Storelli G,** Defaye A, Erkosar B, Hols P, Royet J, Leulier F (2011) Lactobacillus plantarum promotes Drosophila systemic growth by modulating hormonal signals through TOR-dependent nutrient sensing. *Cell metabolism* **14**:403-14 https://doi.org/10.1016/j.cmet.2011.07.012

**Tazume S,** Umehara K, Matsuzawa H, Aikawa H, Hashimoto K, Sasaki S (1991) Effects of germfree status and food restriction on longevity and growth of mice. *Jikken dobutsu Experimental animals* **40**:517-22

**R Core Team** (2024) R: A language and environment for statistical computing.

**Toprak U,** Hegedus D, Doğan C, Güney G (2020) A journey into the world of insect lipid metabolism. *Arch Insect Biochem Physiol* **104**:e21682 https://doi.org/10.1002/arch.21682

**Turnbaugh PJ,** Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* **444**:1027 https://doi.org/10.1038/nature05414

**Valenzano D,** Benayoun BA, Singh P, Zhang E, Etter PD, Hu C-K, Clément-Ziza M, Willemsen D, Cui R, Harel I, *et al.* (2015) The African Turquoise Killifish Genome Provides Insights into Evolution and Genetic Architecture of Lifespan. *Cell* **163**:1539-1554 https://doi.org/10.1016/j.cell.2015.11.008

**Vijay-Kumar M,** Aitken JD, Carvalho FA, Cullender TC, Mwangi S, Srinivasan S, Sitaraman SV, Knight R, Ley RE, Gewirtz AT (2010) Metabolic Syndrome and Altered Gut Microbiota in Mice Lacking Toll-Like Receptor 5. *Science* **328**:228-231 https://doi.org/10.1126/science.1179721

**Wang Y,** Staubach F (2018) Individual variation of natural D.melanogaster-associated bacterial communities. *FEMS Microbiol Lett* **365**:fny017 https://doi.org/10.1093/femsle/fny017

**Watnick PI,** Jugder B-E (2020) Microbial Control of Intestinal Homeostasis via Enteroendocrine Cell Innate Immune Signaling. *Trends Microbiol* **28**:141-149 https://doi.org/10.1016/j.tim.2019.09.005

**Wilkinson JE,** Burmeister L, Brooks SV, Chan C-C, Friedline S, Harrison DE, Hejtmancik JF, Nadon N, Strong R, Wood LK, *et al.* (2012) Rapamycin slows aging in mice: Rapamycin slows aging in mice. *Aging Cell* **11**:675-682 https://doi.org/10.1111/j.1474-9726.2012.00832.x

**Williams GC** (1957) Pleiotropy, natural selection, and the evolution of senescence. *Evolution* **11**

**Wong A,** Dobson AJ, Douglas AE (2014) Gut microbiota dictates the metabolic response of Drosophila to diet. *The Journal of Experimental Biology* **217**:1894-1901 https://doi.org/10.1242/jeb.101725

**Wong AC-N,** Chaston JM, Douglas AE (2013) The inconstant gut microbiota of Drosophila species revealed by 16S rRNA gene analysis. *ISME J* **7**:1922-1932 https://doi.org/10.1038/ismej.2013.86

**Wong AC-N,** Dobson AJ, Douglas AE (2014) Gut microbiota dictates the metabolic response of Drosophila to diet. *J Exp Biology* **217**:1894-1901 https://doi.org/10.1242/jeb.101725

**Zane F,** Bouzid H, Marmol SS, Brazane M, Besse S, Molina JL, Cansell C, Aprahamian F, Durand S, Ayache J, *et al.* (2023) Smurfness-based two-phase model of ageing helps deconvolve the ageing transcriptional signature. *Aging Cell* **22**:e13946 https://doi.org/10.1111/acel.13946

**Zhang A,** Meecham-Garcia G, Hong CN, Xie P, Kern CC, Zhang B, Chapman H, Gems D (2024) Characterization of Effects of mTOR Inhibitors on Aging in Caenorhabditis elegans. *J Gerontol, Ser A: Biol Sci Méd Sci* **79**:glae196 https://doi.org/10.1093/gerona/glae196

# Peer reviews

## Reviewer #1 (Public review):

Summary:

In this study the authors use a Drosophila model to demonstrate that Tachykinin (Tk) expression is regulated by the microbiota. In Drosophila conventionally reared (CR) flies are typically shorter lived than those raised without a microbiota (axenic). Here, knockdown of Tk expression is found to prevent lifespan shortening by the microbiota and the reduction of lipid stores typically seen in CR flies when compared to axenic counterparts. It does so without reducing food intake or fecundity which are often seen as necessary trade-offs for lifespan extension. Further, the strength of the interaction between Tk and the microbiota is found to be bacteria specific and is stronger in Acetobacter pomorum (Ap) mono-associated flies compared to Levilactobacillus brevis (Lb) mono-association. The impact on lipid storage was also only apparent in Ap-flies.

Building on these findings the authors show that gut specific knockdown is largely sufficient to explain these phenotypes. Knockdown of the Tk receptor, TkR99D, in neurons recapitulates the lifespan phenotype of intestinal Tk knockdown supporting a model whereby Tk from the gut signals to TkR99D expressing neurons to regulate lifespan. In addition, the authors show that FOXO may have a role in lifespan regulation by the Tk-microbiota interaction. However, they rule out a role for insulin producing cells or Akh-producing cells suggesting the microbiota-Tk interaction regulates lifespan through other, yet unidentified, mechanisms.

Major comments:

Overall, I find the key conclusions of the paper convincing. The authors present an extensive amount of experimental work, and their conclusions are well founded in the data. In particular, the impact of TkRNAi on lifespan and lipid levels, the central finding in this study, has been demonstrated multiple times in different experiments and using different genetic tools. As a result, I don't feel that additional experimental work is necessary to support the current conclusions.

However, I find it hard to assess the robustness of the lifespan data from the other manipulations used (TkR99DRNAi, TkRNAi in dFoxo mutants etc.) because information on the population size and whether these experiments have been replicated is lacking. Can the authors state in the figure legends the numbers of flies used for each lifespan and whether replicates have been done? For all other data it is clear how many replicates have been done, and the methods give enough detail for all experiments to be reproduced.

Significance:

Overall, I find the key conclusions of the paper convincing. The authors present an extensive amount of experimental work, and their conclusions are well founded in the data. We have known that the microbiota influence lifespan for some time but the mechanisms by which they do so have remained elusive. This study identifies one such mechanism and as a result opens several avenues for further research. The Tk-microbiota interaction is shown to be important for both lifespan and lipid homeostasis, although it's clear these are independent

phenotypes. The fact that the outcome of the Tk-microbiota interaction depends on the bacterial species is of particular interest because it supports the idea that manipulation of the microbiota, or specific aspects of the host-microbiota interaction, may have therapeutic potential.

These findings will be of interest to a broad readership spanning host-microbiota interactions and their influence on host health. They move forward the study of microbial regulation of host longevity and have relevance to our understanding of microbial regulation of host lipid homeostasis. They will also be of significant interest to those studying the mechanisms of action and physiological roles of Tachykinins.

Field of expertise: Drosophila, gut, ageing, microbiota, innate immunity

https://doi.org/10.7554/eLife.109547.1.sa3

## Reviewer #2 (Public review):

Summary:

The main finding of this work is that microbiota impacts lifespan though regulating the expression of a gut hormone (Tk) which in turn acts on its receptor expressed on neurons. This conclusion is robust and based on a number of experimental observations, carefully using techniques in fly genetics and physiology: 1) microbiota regulates Tk expression, 2) lifespan reduction by microbiota is absent when Tk is knocked down in gut (specifically in the EEs), 3) Tk knockdown extends lifespan and this is recapitulated by knockdown of a Tk receptor in neurons. These key conclusions are very convincing. Additional data are presented detailing the relationship between Tk and insulin/IGF signalling and Akh in this context. These are two other important endocrine signalling pathways in flies. The presentation and analysis of the data are excellent.

There are only a few experiments or edits that I would suggest as important to confirm or refine the conclusions of this manuscript. These are:

(1) When comparing the effects of microbiota (or single bacterial species) in different genetic backgrounds or experimental conditions, I think it would be good to show that the bacterial levels are not impacted by the other intervention(s). For example, the lifespan results observed in Figure 2A are consistent with Tk acting downstream of the microbes but also with Tk RNAi having an impact on the microbiota itself. I think this simple, additional control could be done for a few key experiments. Similarly, the authors could compare the two bacterial species to see if the differences in their effects come from different ability to colonise the flies.

(2) The effect of Tk RNAi on TAG is opposite in CR and Ax or CR and Ap flies, and the knockdown shows an effect in either case (Figure 2E, Figure 3D). Why is this? Better clarification is required.

(3) With respect to insulin signalling, all the experiments bar one indicate that insulin is mediating the effects of Tk. The one experiment that does not is using dilpGS to knock down TkR99D. Is it possible that this driver is simply not resulting in an efficient KD of the receptor? I would be inclined to check this, but as a minimum I would be a bit more cautious with the interpretation of these data.

(4) Is it possible to perform at least one lifespan repeat with the other Tk RNAi line mentioned? This would further clarify that there are no off-target effects that can account for the phenotypes.

There are a few other experiments that I could suggest as I think they could enrich the current manuscript, but I do not believe they are essential for publication:

(5) The manuscript could be extended with a little more biochemical/cell biology analysis. For example, is it possible to look at Tk protein levels, Tk levels in circulation, or even TkR receptor activation or activation of its downstream signalling pathways? Comparing Ax and CR or Ap and CR one would expect to find differences consistent with the model proposed. This would add depth to the genetic analysis already conducted. Similarly, for insulin signalling - would it be possible to use some readout of the pathway activity and compare between Ax and CR or Ap and CR?

(6) The authors use a pan-acetyl-K antibody but are specifically interested in acetylated histones. Would it be possible to use antibodies for acetylated histones? This would have the added benefit that one can confirm the changes are not in the levels of histones themselves.

(7) I think the presentation of the results could be tightened a bit, with fewer sections and one figure per section.

Significance:

The main contribution of this manuscript is the identification of a mechanism that links the microbiota to lifespan. This is very exciting and topical for several reasons:

(1) The microbiota is very important for overall health but it is still unclear how. Studying the interaction between microbiota and health is an emerging, growing field, and one that has attracted a lot of interest, but one that is often lacking in mechanistic insight. Identifying mechanisms provides opportunities for therapies. The main impact of this study comes from using the fruit fly to identify a mechanism.

(2) It is very interesting that the authors focus on an endocrine mechanism, especially with the clear clinical relevance of gut hormones to human health recently demonstrated with new, effective therapies (e.g. Wegovy).

(3) Tk is emerging as an important fly hormone and this study adds a new and interesting dimension by placing TK between microbiota and lifespan.

I think the manuscript will be of great interest to researchers in ageing, human and animal physiology and in gut endocrinology and gut function.

https://doi.org/10.7554/eLife.109547.1.sa2

## Reviewer #3 (Public review):

Summary:

Marcu et al. demonstrate a gut-neuron axis that is required for the lifespan-shortening effects mediated by gut bacteria. They show that the presence of commensal bacteria-particularly Acetobacter pomorum-promotes Tk expression in the gut, which then binds to neuronal tachykinin receptors to shorten lifespan. Tk has also recently been reported to extend lifespan via adipokinetic hormone (Akh) signaling (Ahrentløv et al., Nat Metab 7, 2025), but the mechanism here appears distinct. The lifespan shortening by Ap via Tk seems to be partially dependent on foxo and independent of both insulin signaling and Akh-mediated lipid mobilization.

Although the detailed mechanistic link to lifespan is not fully resolved, the experiment and its results clearly show the involvement of the molecules tested. This work adds a valuable

dimension to our growing understanding of how gut bacteria influence host longevity. However, there are some points that should be addressed.

(1) Tk+ EEC activity should be assessed directly, rather than relying solely on transcript levels. Approaches such as CaLexA or GCaMP could be used.

(2) In Line243, the manuscript states that the reporter activity was not increased in the posterior midgut. However, based on the presented results in Fig4E, there is seemingly not apparent regional specificity. A more detailed explanation is necessary.

(3) If feasible, assessing foxo activation would add mechanistic depth. This could be done by monitoring foxo nuclear localization or measuring the expression levels of downstream target genes.

(4) Fig1C uses Adh for normalization. Given the high variability of the result, the authors should (1) check whether Adh expression levels changed via bacterial association and/or (2) compare the results using different genes as internal standard.

(5) While the difficulty of maintaining lifelong axenic conditions is understandable, it may still be feasible to assess the induction of Tk (i.e.. Tk transcription or EE activity upregulation) by the microbiome on males.

(6) We also had some concerns regarding the wording of the title.

Fig6B and C suggests that TkR86C, in addition to TkR99D, may be involved in the A. pomorum-lifespan interaction. Consider revising the title to refer more generally to the "tachykinin receptor" rather than only TkR99D.

The difference between "aging" and "lifespan" should also be addressed. While the study shows a role for Tk in lifespan, assessment of aging phenotypes (e.g. Climbing assay, ISC proliferation) beyond the smurf assay is required to make conclusions about aging.

(7) The statement in Line 82 that EEs express 14 peptide hormones should be supported with an appropriate reference, if available.

Significance:

General assessment: The main strength of this study is the careful and extensive lifespan analyses, which convincingly demonstrate the role of gut microbiota in regulating longevity. The authors clarify an important aspect of how microbial factors contribute to lifespan control. The main limitation is that the study primarily confirms the involvement of previously reported signaling pathways, without identifying novel molecular players or previously unrecognized mechanisms of lifespan regulation.

Advance: The lifespan-shortening effect of Acetobacter pomorum (Ap) has been reported previously, as has the lifespan-shortening effect of Tachykinin (Tk). However, this study is the first to link these two factors mechanistically, which represents a significant and original contribution to the field. The advance is primarily mechanistic, providing new insight into how microbial cues converge on host signaling pathways to influence ageing.

Audience: This work will be of particular interest to a specialized audience of basic researchers in ageing biology. It will also attract interest from microbiome researchers who are investigating host-microbe interactions and their physiological consequences. The findings will be useful as a conceptual framework for future mechanistic studies in this area.

Field of expertise: Drosophila ageing, lifespan, microbiome, metabolism

https://doi.org/10.7554/eLife.109547.1.sa1

## Author response:

**(1) General Statements**

The goal of our study was to mechanistically connect microbiota to host longevity. We have done so using a combination of genetic and physiological experiments, which outline a role for a neuroendocrine relay mediated by the intestinal neuropeptide *Tachykinin*, and its receptor *TkR99D* in neurons. We also show a requirement for these genes in metabolic and healthspan effects of microbiota.

The referees' comments suggest they find the data novel and technically sound. We have added data in response to numerous points, which we feel enhance the manuscript further, and we have clarified text as requested. Reviewer #3 identified an error in Figure 4, which we have rectified. We felt that some specific experiments suggested in review would not add significant further depth, as we articulate below.

Altogether our reviewers appear to agree that our manuscript makes a significant contribution to both the microbiome and ageing fields, using a large number of experiments to mechanistically outline the role(s) of various pathways and tissues. We thank the reviewers for their positive contributions to the publication process.

**(2) Description of the planned revisions**

> ***Reviewer #2:***
>
> *Not...essential for publication...is it possible to look at Tk protein levels?*

We have acquired a small amount of anti-TK antibody and we will attempt to immunostain guts associated with *A. pomorum* and *L. brevis*. We are also attempting the equivalent experiment in mouse colon reared with/without a defined microbiota. These experiments are ongoing, but we note that the referee feels that the manuscript is a publishable unit whether these stainings succeed or not.

**(3) Description of the revisions that have already been incorporated in the transferred manuscript**

> ***Reviewer #1:***
>
> *Can the authors state in the figure legends the numbers of flies used for each lifespan and whether replicates have been done?*

We have incorporated the requested information into legends for lifespan experiments.

> *Do the interventions shorten lifespan relative to the axenic cohort? Or do they prevent lifespan extension by axenic conditions? Both statements are valid, and the authors need to be consistent in which one they use to avoid confusing the reader.*

We read these statements differently. The only experiment in which a genetic intervention prevented lifespan extension by axenic conditions is neuronal *TkR86C* knockdown (Figure 6B-C). Otherwise, microbiota shortened lifespan relative to axenic conditions, and genetic knockdowns extend blocked this effect (e.g. see lines 131-133). We have ensured that the framing is consistent throughout, with text edited at lines 198-199, 298-299, 311-312, 345-347, 407-408, 424-425, 450, 497-503.

> *TkRNAi consistently reduces lipid levels in axenic flies (Figs 2E, 3D), essentially phenocopying the loss of lipid stores seen in control conventionally reared (CR) flies relative to control axenic. This suggests that the previously reported role of Tk in lipid storage - demonstrated through increased lipid levels in TkRNAi flies (Song et al (2014)*

> *Cell Rep 9(1): 40) - is dependent on the microbiota. In the absence of the microbiota TkRNAi reduces lipid levels. The lack of acknowledgement of this in the text is confusing*

We have added text at lines 219-222 to address this point. We agree that this effect is hard to interpret biologically, since expressing RNAi in axenics has no additional effect on *Tk* expression (Figure S7). Consequently we can only interpret this unexpected effect as a possible off-target effect of RU feeding on TAG, specific to axenic flies. However, this possibility does not void our conclusion, because an off-target dimunition of TAG cannot explain why CR flies accumulate TAG following $Tk^{RNAi}$ induction. We hope that our added text clarifies.

> *I have struggled to follow the authors logic in ablating the IPCs and feel a clear statement on what they expected the outcome to be would help the reader.*

We have added the requested statement at lines 423-424, explaining that we expected the IPC ablation to render flies constitutively long-lived and non-responsive to *A pomorum*.

> *Can the authors clarify their logic in concluding a role for insulin signalling, and qualify this conclusion with appropriate consideration of alternative hypotheses?*

We have added our logic at lines 449-454. In brief, we conclude involvement for insulin signalling because FoxO mutant lifespan does not respond to $Tk^{RNAi}$, and diminishes the lifespan-shortening effect of *A. pomorum*. However, we cannot state that the effects are direct because we do not have data that mechanistically connects Tk/TkR99D signalling directly in insulin-producing cells. The current evidence is most consistent with insulin signalling priming responses to microbiota/Tk/TkR99D, as per the newly-added text.

> *Typographical errors*

We have remedied the highlighted errors, at lines 128-140.

> ***Reviewer #2:***
>
> *it would be good to show that the bacterial levels are not impacted [by TkRNAi]*

We have quantified CFUs in CR flies upon ubiquitous TkRNAi (Figure S5), finding that the RNAi does not affect bacterial load. New text at lines 138-139 articulates this point.

> *The effect of Tk RNAi on TAG is opposite in CR and Ax or CR and Ap flies, and the knockdown shows an effect in either case (Figure 2E, Figure 3D). Why is this?*

As per response to Reviewer #1, we have added text at lines 219-222 to address this point.

> *Is it possible to perform at least one lifespan repeat with the other Tk RNAi line mentioned?*

We have added another experiment showing longevity upon knockdown in conventional flies, using an independent *TkRNAi* line (Figure S3).

> ***Reviewer #3:***
>
> *In Line243, the manuscript states that the reporter activity was not increased in the posterior midgut. However, based on the presented results in Fig4E, there is seemingly not apparent regional specificity. A more detailed explanation is necessary.*

We thank the reviewer sincerely for their keen eye, which has highlighted an error in the previous version of the figure. In revisiting this figure we have noticed, to our dismay, that the figures for GFP quantification were actually re-plots of the figures for (ac)K quantification. This error led to the discrepancy between statistics and graphics, which

thankfully the reviewer noticed. We have revised the figure to remedy our error, and the statistics now match the boxplots and results text.

> *Fig1C uses Adh for normalization. Given the high variability of the result, the authors should (1) check whether Adh expression levels changed via bacterial association*

We selected Adh on the basis of our RNAseq analysis, which showed it was not different between AX and CV guts, whereas many commonly-used "housekeeping" genes were. We have now added a plot to demonstrate (Figure S2).

> *The statement in Line 82 that EEs express 14 peptide hormones should be supported with an appropriate reference*

We have added the requested reference (Hung et al, 2020) at line 86.

**(4) Description of analyses that authors prefer not to carry out**

> ***Reviewer #1:***
>
> *I'd encourage the authors to provide lifespan plots that enable comparison between all conditions*

We have avoided this approach because the number of survival curves that would need to be presented on the same axis (e.g. 16 for Figure 5) is not legible. However we have ensured that axes on faceted plots are equivalent and with grid lines for comparison. Moreover, our approach using statistical coefficients (EMMs) enables direct quantitative comparison of the differences among conditions.

> ***Reviewer #2:***
>
> *Is it possible that this driver is simply not resulting in an efficient KD of the receptor? I would be inclined to check this*

This comment relates to Figure 7G. We do see an effect of the knockdown in this experiment, so we believe that the knockdown is effective. However the direction of response is not consistent with our hypothesis so the experiment is not informative about the role of these cells. We therefore feel there is little to be gained by testing efficacy of knockdown, which would also be technically challenging because the cells are a small population in a larger tissue which expresses the same transcripts elsewhere (i.e. necessitating FISH).

> *Would it be possible to use antibodies for acetylated histones?*

The comment relates to Figure 4C-E. The proposed studies would be a significant amount of work because, to our knowledge, the specific histone marks which drive activation in TK+ cells remain unknown. On the other hand, we do not see how this information would enrich the present story, rather such experiments would appear to be the beginning of something new. We therefore agree with Reviewer #1 (in cross-commenting) that this additional work is not justified.

> ***Reviewer #3:***
>
> *Tk+ EEC activity should be assessed directly, rather than relying solely on transcript levels. Approaches such as CaLexA or GCaMP could be used.*

We agree with reviewers 1-2 (in cross-commenting) that this proposal is non-trivial and not justified by the additional insight that would be gained. As described above, we are attempting to immunostain Tk, which if successful will provide a third line of evidence for regulation of Tk+ cells. However we note that we already have the strongest possible evidence for a role of these cells via genetic analysis (Figure 5).

> *While the difficulty of maintaining lifelong axenic conditions is understandable, it may still be feasible to assess the induction of Tk (ie. Tk transcription or EE activity upregulation) by the microbiome on males.*

As the reviewer recognises, maintaining axenic experiments for months on end is not trivial. Given the tendency for males either to simply mirror female responses to lifespan-extending interventions, or to not respond at all, we made the decision in our work to only study females. We have instead emphasised in the manuscript that results are from female flies.

> *TkR86C, in addition to TkR99D, may be involved in the A. pomorum-lifespan interaction. Consider revising the title to refer more generally to the "tachykinin receptor" rather than only TkR99D.*

We disagree with this interpretation: the results do not show that *TkR86C-RNAi* recapitulates the effect of enteric *Tk-RNAi*. A potentially interesting interaction is apparent, but the data do not support a causal role for *TkR86C*. A causal role is supported only for *TkR99D*, knockdown of which recapitulates the longevity of axenic flies and $Tk^{RNAi}$ flies_._ Therefore we feel that our current title is therefore justified by the data, and a more generic version would misrepresent our findings.

> *The difference between "aging" and "lifespan" should also be addressed.*

The smurf phenotype is a well-established metric of healthspan. Moreover, lifespan is the leading aggregate measure of ageing. We therefore feel that the use of “ageing” in the title is appropriate.

> *If feasible, assessing foxo activation would add mechanistic depth. This could be done by monitoring foxo nuclear localization or measuring the expression levels of downstream target genes.*

Foxo nuclear localisation has already been shown in axenic flies (Shin et al, 2011). We have added text and citation at lines 401-402.

https://doi.org/10.7554/eLife.109547.1.sa0