Arc1 and the microbiota together modulate growth and metabolic traits in *Drosophila*

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I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. In particular, there are some issues with how some experiments have been conducted and quantified in the current version of the manuscript, and it will also be important to establish the tissue cell type from which Arc1 exerts its microbiota-related effects. If you are able to revise the manuscript along the lines suggested, which will involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing

how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This is a timely and well executed study revealing the interdependent influence of a host genotype, nutrition and microbiota on Drosophila developmental growth. Specifically, through a loss of function approach the authors establish that the gene encoding Arc1, a Drosophila homolog of mammalian Arc/arg3.1 a master regulator of synaptic plasticity in the brain, functionally interact with nutrients, a microbiota member and Insulin signaling to support developmental growth.

This work does not fall into the classical limitations of Drosophila microbiome studies as the authors pay serious attention to control with the best experimental practice of the field their microbiome-related assays as such they establish a robust microbiota-related phenotype of Arc1 null mutant flies. Therefore the microbe-related experiments are very robust and conclusive. Also I was very please to see the authors testing multiple alleles of Arc1 as well as transheterozygous. The genetics is solid here.

Comments for the author

That said, the authors do not embark on testing how Arc1 influence developmental growth. In an attempt in that direction I feel it is important to rescue the mutant line with a ubiquitously expressed transgene as well as probing in which tissue Arc1 is required to mediate developmental growth.
Studies have started dissecting Arc1 function in the brain or the NMJ and the authors report that Arc1 expression in adults is influenced by the microbiota, ie Arc1 is upregulated in the head of GF flies and down in GF adult guts. What about larval brains and larval guts? Is Arc1 expression in these tissues also influenced by diet changes or just the microbial environment? The authors should test rescuing Arc1 expression in Arc1 mutants in these tissues.
Also the authors quickly discuss Arc1 expression in the Prothoracic gland, is Arc1 important in this tissue to support developmental growth? Is Arc1 expressed in Ilps expressing neurons of the larval brain?
Finally, the connection with IIS should be strengthen. Are dILPs retained or depleted in Ilps expressing neurons of the Arc1 mutant larval brains? Can the author test additional IIS molecular read-out beyond tGPH reporter which is not a very sensitive IIS read-out.
I feel the result of these experiments will convey a more complete picture about how Arc1 may influence developmental phenotypes in relation to Insulin signaling, nutrients and microbes, yet I am positively impressed by the work.
François Leulier

Reviewer 2

Advance summary and potential significance to field

The manuscript of Keith et al. describes how Drosophila-associated bacteria species contribute to larval growth on several distinct diets. Two of the authors' interesting findings were that (1) Arc1 mutant larvae exhibit a marked delay in pupariation, compared to wild-type animals, under a germ-free (GF) condition (the phenotype in developmental timing in GF; Figure 1B and C) and (2) transplantation with Acetobacter sp. alone totally cancels this phenotype (Figure 2A-D). Arc1 is an evolutionally conserved gene and plays a pivotal role in regulation of synaptic plasticity. To unravel mechanisms underlying the timing phenotype and the effect of Acetobacter sp., authors performed three-way analysis: the genotype of larvae (wild-type or Arc1 mutant), GF or gnotobiotic/GNO, and the diets.

Comments for the author

Below extracted are key statements either in the abstract or in the main text, together with relevant figure numbers, to connect with major comments, because important questions remain regarding every statement:

1. Arc1 exhibits tissue-specific microbiota-dependent expression changes (Figure 1A).

2. Arc1 mutant GF larvae undergo substantially prolonged L1 and L2 phases (Figure 2C).

3. Monoassociation with a single Acetobacter sp. isolate was sufficient to enable Arc1 mutants to develop at a wild-type rate (Figure 2A-D).

4. These developmental phenotypes are highly sensitive to composition of the larval diet (Figure 3).

5. GF Arc1 mutants display multiple traits consistent with reduced insulin signaling activity that are reverted by association with Acetobacter sp. (Figure 5).

Major comments
Related to the authors' statements #1, #2 and #3:
Which larval tissue and cell type requires Arc1 to promote growth and pupariation of GF animals? The expression analysis of Figure 1A was done only in adult tissues. The authors briefly state expression in larvae, which was done by others: "Arc1 is strongly expressed in one small cluster of large neurons in each lobe of both the larval brain and the pars cerebralis region of the adult brain that are proximal to and partially overlap with the insulin producing cells (IPCs; Mattaliano et al., 2007; Mosher et al., 2015). Further, Mattaliano et al. (2007) found that selectively restoring Arc1 expression in the IPCs was sufficient to revert the starvation resistance of Arc1 mutants." According to FlyBase, Arc1 expression is extremely high in the digestive system and very high in imaginal tissues in wandering L3 larvae, and is detected also in embryo, L1 and L2 (moderate, moderate and low, respectively).

Obvious questions and additional experiments include the following:
- Which tissue and cell type express Arc1 in L1, L2 and L3? The authors can use at least Arc1-Gal4 to address this question. It is preferable to examine how much this Gal4 driver faithfully recapitulates the endogenous Arc1 expression at the larval stages.
- What is the relevance of the microbiota-dependent expression change of Arc1 in this study? This reviewer is asking this question because the hypothetical role of Arc1 in larval growth and pupariation becomes totally dispensable when larvae are transplanted with Acetobacter sp.
- Which tissue or cell type requires Arc1 function to promote larval growth and pupariation under the GF condition? This question can be addressed by restoring Arc1 expression in cell-type selective manners (IPC, motor neuron, pan-neuron, ring gland, gut, etc.) in the GF mutant larvae and examine whether the developmental timing becomes indistinguishable to that of wild-type GF larvae. This reviewer understands that a substantial amount of work is necessary to complete this experiment; however, the aforementioned question is directly related to the potential significance of this study and probably one of the most important questions. Even if Arc1 is actually required in broad tissues, other important questions are what is the role of Arc1 in those tissues and how that role becomes dispensable in the presence of Acetobacter sp. (see also comments related to the authors' statements #5).

Related to the authors' statement #4:
It is difficult to understand the section "Host diet can modulate the Arc1-dependent GF larval developmental delay":
- Results of Figure 3 are complicated and difficult to interpret. For example, under two of the three high-yeast conditions (10% yeast-3% or 5% glucose), the contribution of Acetobacter sp. is negligible and what seems critical is the genotype of the host (wild type or Arc1 mutant). However, the differential effect of the genotypes is marginal. What does this result tell us about the role of Arc1 in GF larvae?
- It has been well known that yeast is a rich source of amino acids and many other nutrients, including unknown but critical to larval growth (see Figure 5a in Piper et al. Nature Methods, 2014). This section, in particular the paragraph of line 260-279, can be trimmed down.

Related to the authors' statement #5:

- Do the authors state "GF Arc1 mutants display multiple traits consistent with reduced insulin signaling activity" compared to the GF wild type? Or do the authors compare the activity between the GF Arc1 mutant and the other three conditions (see also next)? Whichever the case, evidence of the tGPH localization is not convincing (Figure 5F) and some quantification may be necessary. Additional evidence regarding either a subcellular localization of FoxO, expression of dlips, or the phosphor-Akt level would be needed to strengthen the statement.
- In Figure 5A, what is the basis of the illustrations of "Reduced IIS" and "Over-activated IIS"? Cite at least representative original papers (not reviews), which give evidence for each illustration, in the legend. Again, specify which comparison the authors are discussing, because "smaller cells," "larger cells," "more cells," etc are all confusing.
- The authors discuss the data of Figure 5B and eventually stated "Arc1 may also contribute to wing growth through unknown additional mechanisms" (line 378). What mechanism increases the wing cell number of the Arc1 mutant irrespective of Acetobacter sp remains to be solved. This reviewer suspects the possibility that Arc1 is in fact expressed in the wing imaginal disc (see modENCODE Anatomy RNA-seq in FlyBase) and contributes to its tissue growth in a cell-autonomous manner.

Reviewer 3

Advance summary and potential significance to field

Here Keith et al., describe a potential role for Arc1 in regulating metabolic homeostasis through insulin signaling, in response to the nutritional and microbial content of the Drosophila diet. Their main finding is that developmental defects of arc1 mutants can be rescued by supplementing diets with specific live bacteria.
This study is relevant since looks into mechanistic coupling between external environment, the flies'
physiology and progress through development. Despite the excitement for such a mechanism, in its current form, the manuscript falls short of effectively supporting its main conclusions. Below I outline a few major issues that need to be addressed in order to support the authors' conclusions.

Comments for the author

1- One criticism is that the experiments were performed at 23˚C rather 25˚C, the optimal developmental temperature for larvae. This lower temperature extends larval development to 7 days, instead of the standard 5. (1 embryonic, plus 4 larval). Additionally, the fact that egg collections/1st instar larvae don't seem to be controlled so that the same number of larvae grow in the vial (10-40 is too much variation, all should around 40-50 ±1, depending on the size of the vials used). Larval density can highly influence developmental and growth rates. Hence it is hard to evaluate with confidence the data presented. All experiments should be performed at 25˚C in a humidified incubator with essentially the exact same number of L1s transferred into vials after short egg collections (usually 4-6 hours).

2- 40 µL of O/N culture of bacteria doesn't seem to be a precise measure for how much bacteria are supplemented to the diets. Whether dead or alive, bacterial cultures should be grown to the same density and the same density used each time for each and all experiments.

3- Arc1 neuronal transcripts were identified as microbiota-responsive. What happens to Arc1 protein? Please test and show if Arc1 protein levels are also differentially regulated in GF flies.

4- On line 278-9, the authors propose that differences in development observed in the different diets could be due to differences in the levels of bacterial growth on such diets. It is crucial to test this hypothesis and test if indeed bacterial growth is influenced by the different fly diets and if this is an explanation for the changes observed.

5- What is the result of supplementing Arc1E8 mutants with dead bacteria plus acetic acid?

6- Feeding assays were performed for too long. A 3 hour feeding assay saturates guts and any difference in feeding would not be detected. Feeding assays during pre-wandering larval development should be done for 20-30 minutes at the most.

7- "Strongly reduced" localization of the tGPH reporter in Figure 5F is hard to visualize, please quantify (and please keep in mind that focal plane and how these fat bodies are mounted can influence the perception of membrane versus nuclear localization), AND use another approach to measure levels of insulin signaling (westerns for SK6 levels or immunostaining for FOXO subcellular localization, for example). This connection with IIS is weak and needs further support since strong conclusions are being derived from these data.

8- Clonal analysis using wing discs is a better and more accurate approach to conclude what is happening to cellular proliferation and growth. Again, here, growth conditions (temperature, humidity and larval density in vials) have to be more tightly controlled.

9- What are the effects of overexpressing Arc1 in GF wild-type larvae? Is Arc1 sufficient to rescue the prolonged larval development and stunted growth observed in wild-type germ-free larvae? What about in Arc1E8 GF mutants? Please test.

10- Conclusions drawn in the discussion have to be toned down to more accurately represent the results.

Minor concerns to address:
a) The link between Arc1 and Il2 signaling is not novel. Mattaliano et al, observed a link between Arc1-expressing cells and IPCs and Mosher et al, proposed that Arc1 regulation is downstream of Ilps. The present findings should be better described in the context of these prior findings throughout the paper and especially in the Discussion.
b) Line 285-7: enhanced starvation survival could be solely due to the observed increase in fat levels for the mutants, please also mention this possibility.
c) Figures 3 and 4 are too small to read as presented.
d) Add reference to figure 5E on line 382.

First revision

Author response to reviewers' comments

We are grateful to each of the reviewers for providing thoughtful comments, criticisms, and suggestions which we feel have substantially improved our manuscript.

Points 1-3: Reviewers1-3

1) Tissue requirements of Arc1 for developmental timing in GF larvae
Reviewer 1:
"…the authors do not embark on testing how Arc1 influence developmental growth. In an attempt in that direction I feel it is important to rescue the mutant line with a ubiquitously expressed transgene as well as probing in which tissue Arc1 is required to mediate developmental growth… the authors report that Arc1 expression in adults is influenced by the microbiota, ie Arc1 is upregulated in the head of GF flies and down in GF adult guts. What about larval brains and larval guts?...The authors should test rescuing Arc1 expression in Arc1 mutants in these tissues… Also the authors quickly discuss Arc1 expression in the Prothoracic gland, is Arc1 important in this tissue to support developmental growth?"

Reviewer 2:
"Which larval tissue and cell type requires Arc1 to promote growth and pupariation of GF animals?...This question can be addressed by restoring Arc1 expression in cell-type selective manners (IPC, motor neuron, pan-neuron, ring gland, gut, etc.) in the GF mutant larvae and

examine whether the developmental timing becomes indistinguishable to that of wild-type GF larvae…the aforementioned question is directly related to the potential significance of this study and probably one of the most important questions."

Reviewer 3:
"9-What are the effects of overexpressing Arc1 in GF wild-type larvae? Is Arc1 sufficient to rescue the prolonged larval development and stunted growth observed in wild-type germ-free larvae? What about in Arc1E8 GF mutants? Please test."

Author response:
Each of the reviewers asked that we examine the tissue-specific requirements of *Arc1* in the context of GF growth and metabolic phenotypes. We strongly agree that this is essential to gain a better understanding of how the Arc1-microbiota collaboration impacts host physiology. To address this, we performed a series of genetic rescue experiments, the results of which are presented in Fig. 6, S6, and S8. Specifically, we expressed a previously described *UAS-Arc1* transgene (Mattaliano et al. 2007) in wild type and/or the $Arc1^{E8}$ mutant background using a variety of GAL4 drivers, and measured time to pupariation under GF conditions. We selected drivers that express in cells and tissues with well- established roles in developmental growth, and/or where *Arc1* is expressed. These drivers included (1) ubiquitous, (2) pan-neuronal, (3) IPCs, (4) prothoracic gland, (5) foregut/midgut, (6) midgut/hindgut/malpigian tubules, (7) somatic muscle, and (8) fat body. We did not use the existing *Arc1-GAL4* driver for these experiments, and our rationale is explained in the next section.

We found that ubiquitous expression, IPC specific, or prothoracic gland specific expression of *Arc1* all partially **suppressed** the developmental delay of GF $Arc1^{E8}$ animals (Fig. 6A-C,S6A). Expression of *Arc1* using these drivers did not impact the growth rate of wild type GF animals (Fig. S8A-D). Interestingly, pan-neuronal or foregut/midgut expression of *Arc1* in GF animals either $Arc1^{E8}$ mutant or wild type further **enhanced** the developmental delay (Fig. 6A,S6E,F). Further, we demonstrate that IPC specific *Arc1* expression in $Arc1^{E8}$ GF animals suppressed other *Arc1* phenotypes that were only apparent under GF conditions. These additional $Arc1^{E8}$ GF phenotypes included: 1. decreased pupal volume, 2. reduced wing cell size, 3. increased lipid droplet (Fig. 6D-F).

Together our data suggest that *Arc1* expression in multiple tissues is required to fulfill Arc1's function in growth control and metabolism. Given the complexity of *Arc1* expression (see below for more detail), and the systemic nature of growth control and metabolism, this is not surprising. Another consideration for Arc1 function is the 3'UTR. The *UAS-Arc1* rescue transgene we utilized lacks the *Arc1* 3'UTR, and the 3'UTR appears to be important for at least some Arc1 functions (Ashley et al., 2018). Unfortunately, we were unable to obtain the existing *UAS-Arc1* transgenic line that contains the 3'UTR. This will certainly be question for future investigation.

2) Further analysis of Arc1 expression patterns and their potential microbiota sensitivity
Reviewer 1:
"…the authors report that Arc1 expression in adults is influenced by the microbiota, ie Arc1 is upregulated in the head of GF flies and down in GF adult guts. What about larval brains and larval guts? Is Arc1 expression in these tissues also influenced by diet changes or just the microbial environment?"

Reviewer 2:
*"The expression analysis of Figure 1A was done only in adult tissues… Which tissue and cell type express Arc1 in L1, L2 and L3? The authors can use at least Arc1-Gal4 to address this question. It is preferable to examine how much this Gal4 driver faithfully recapitulates the endogenous Arc1 expression at the larval stages…*What is the relevance of the microbiota-dependent expression change of Arc1 in this study? *This reviewer is asking this question because the hypothetical role of Arc1 in larval growth and pupariation becomes totally dispensable when larvae are transplanted with Acetobacter sp."*

Reviewer 3:
"3-Arc1 neuronal transcripts were identified as microbiota-responsive. What happens to Arc1 protein? Please test and show if Arc1 protein levels are also differentially regulated in GF flies."

Author response:
In connection to our investigations of the cell-type requirements for Arc1 function, each of the reviewers also suggested that we examine the microbiota-responsive *Arc1* transcript level changes in greater detail, particularly focusing on the larval stages, and that we test *Arc1* responsiveness to additional variables.

We initially reported that *Arc1* transcript levels change in adult heads and guts of multiple wild-type fly lines grown under GNO and GF conditions (Fig. 1A). To address whether *Arc1* transcript levels respond to nutritional inputs (Reviewer 1), we measured relative *Arc1* mRNA via RT-qPCR in heads and guts of conventionally-reared (i.e. no microbiota manipulation) adult flies either fed or following 24 hr starvation. We find that *Arc1* transcripts significantly decrease in the head following this starvation treatment, but are unaffected in the gut (Fig. 1B). Also as requested (Reviewers 1&2), we examined *Arc1* transcript levels in brain+ring gland and gut samples from GNO and GF feeding third instar larvae; we find a modest, but statistically significant decrease in GF brains, and no difference in the gut (Fig. 2C).

To investigate whether Arc1 protein levels change in response to the microbiota (Reviewer 3), we performed a series of immunostaining experiments to visualize Arc1 expression patterns and level in tissues and stages where we observed *Arc1* transcript changes: adult brain and gut, and larval brain. While quantitative Western blots would have been a better approach to measure tissue-level protein level changes, our attempts to perform Western blots with the Arc1 antibody were unsuccessful. Using immunofluorescence, we discovered changes in both the number of Arc1-positive neurons and the fluorescence intensities in particular sub-populations of Arc1-labelled cells in both adult and larval brains under GF conditions (Fig. 1C,D,S1,2C,D,S3). We also show that the *Arc1* mRNA decrease observed in GF adult guts corresponds to an ~2-fold decrease in Arc1 fluorescence intensity in the midgut (Fig.1E,F). Notably, our study is the first to report Arc1 expression patterns in the adult gut. We think that these experiments have been particularly important, as they reveal cell type specific effects on Arc1 expression from GF rearing that would be completely masked in the tissue-level RT-qPCR analyses.

While Arc1 immunostaining in the larval brain has been published (Mattaliano et al., 2007; Mosher et al., 2015), in GNO animals we carefully documented Arc1 expression in relation to the IPCs (co-labelled with anti-Ilp2; Fig. 2D,S7C-F''). This was previously shown in adult, but not larval brains (Mattaliano et al., 2007). This revealed that Arc1 is weakly expressed in some larval IPCs themselves, in addition to strong expression in neighboring clusters of central brain neurons. In addition, we observed weak Arc1- positive cells adjacent to the IPCs and weak Ilp2-positive cells adjacent to the Arc1-positve neurons. We also show that Arc1 fluorescence intensity in the ring gland significantly increases between the late feeding and wandering third instar larval stages (Fig. S7A-B''). As indicated by the reviewers, these analyses are helpful for the interpretation of our rescue experiments.

As suggested by Reviewer 2, we carefully examined the previously described *Arc1-GAL4* line (Mattaliano et al., 2007) acquired from the Bloomington Stock Center (#37533) by crossing it to *UAS- CD8.ChRFP*. Comparing those expression patterns to those we see with anti-Arc1, we are not confident that this driver fully recapitulates endogenous *Arc1* expression in the larval brain. For this reason, we did not conduct rescue experiments with this driver and we removed data from the original submission reporting developmental rates for *Arc1-GAL4>UAS-reaper* larvae.

3) Additional evidence for connections linking Arc1, host microbial condition, and IIS pathway activity
Reviewer 1:
"…the connection with IIS should be strengthen. Are dILPs retained or depleted in Ilps expressing neurons of the Arc1 mutant larval brains? Can the author test additional IIS molecular read-out beyond tGPH reporter which is not a very sensitive IIS read-out."

Reviewer 2:
"Do the authors state "GF Arc1 mutants display multiple traits consistent with reduced insulin signaling activity" compared to the GF wild type? Or do the authors compare the activity between the GF Arc1 mutant and the other three conditions (see also next)? Whichever the case, evidence of the tGPH localization is not convincing (Figure 5F) and some quantification may be necessary. Additional evidence regarding either a subcellular localization of FoxO, expression of dlips, or the phosphor-Akt level would be needed to strengthen the statement."

Reviewer 3:
"7-'Strongly reduced' localization of the tGPH reporter in Figure 5F is hard to visualize, please quantify (and please keep in mind that focal plane and how these fat bodies are mounted can influence the perception of membrane versus nuclear localization), AND use another approach to measure levels of insulin signaling (westerns for S6K levels or immunostaining for FOXO subcellular localization, for example). This connection with IIS is weak and needs further support since strong conclusions are being derived from these data."

Author response:
We thank the reviewers for their comments and agree that the connection between Arc1, microbes, and IIS activity required more investigation.

We first attempted to quantify membrane *vs.* cytosolic fluorescence intensity of the tGPH reporter under starvation conditions (reduced IIS activity) as a positive control, but we were unable to consistently detect a difference. We are therefore in agreement with the reviewers about the limited sensitivity and utility of this reagent.

Thus, to more thoroughly test the hypothesis that IIS function is perturbed in GF *Arc1* mutants, we conducted a series of additional assays examining IIS signaling at different steps in the pathway for which the fed/starved positive controls yielded the expected differences (data not shown). Some of these specific assays were suggested by the reviewers and included: (1) *Ilp2/3/5* transcript level and (2) Ilp2/5 protein accumulation in the larval brain, and (3) phospho-Akt level, and (4) IIS target gene expression (*InR*, *Thor/4EBP*) in the larval fat body. We also expanded our examination of metabolic effects of *Arc1* and microbe depletion by measuring lipid droplet size in the larval fat body. Finally, we began to examine additional growth control pathways that may be impacted by the Arc1-microbe intersection by measuring relative transcript levels for the biosynthetic pathway genes (Halloween genes) in larval brain and fat body that produce 20E.

These new data are presented in Fig. 7, and are discussed in lines 388-446. In all of the IIS assays we conducted, we discovered changes in *Arc1* mutant animals, the majority of which were only revealed when the *Arc1* mutants were GF. This is consistent with the hypothesis that altered IIS signaling in $Arc1^{E8}$ GF animals is contributing to the larval growth delay, and the other growth and metabolic phenotypes we described. The results of these IIS assays do not point to a simple up or down effect on signaling, however. For example, while pAkt is reduced in $Arc1^{E8}$ GF fat bodies consistent with reduced IIS pathway activity, the effect on the IIS target genes is the opposite of what we would predict from reduced pathway activation (i.e. expression of the negatively regulated target *Thor/4EBP* expression was reduced rather than the predicted increase). These types of contradictions suggested to us that different steps in the pathway are being impacted in different ways either directly, or perhaps indirectly via other growth pathways that are intersecting with Arc1-microbe function. This prompted our examination of the 20E biosynthetic pathway revealing a significant reduction in transcript levels of two genes in the pathway in *Arc1* mutant animals, *sro* only when microbes are depleted, and *shd* regardless of microbial condition. Taken together, our studies reveal a complex set of microbe- dependent and -independent *Arc1* mutant changes in the IIS pathway and the 20E biosynthetic pathway that all may be contributing to the growth phenotypes we observed. A full understanding of precisely how Arc1 and microbes, together with the critical variable of diet, are mechanistically coordinating to regulate host growth and metabolism will likely require years of future work. We believe that we have been appropriately conservative with our conclusions in the manuscript, while simultaneously providing reasonable speculative interpretation of our findings.

Points 4-6: Reviewer 2

4) *"It is difficult to understand the section 'Host diet can modulate the Arc1-dependent GF larval developmental delay':*

- *Results of Figure 3 are complicated and difficult to interpret. For example, under two of the three high- yeast conditions (10% yeast-3% or 5% glucose), the contribution of Acetobacter sp. is negligible and what seems critical is the genotype of the host (wild type or Arc1 mutant). However, the differential effect of the genotypes is marginal. What does this result tell us about the role of Arc1 in GF larvae?*
- *It has been well known that yeast is a rich source of amino acids and many other nutrients, including unknown but critical to larval growth (see Figure 5a in Piper et al. Nature Methods, 2014). This section, in particular the paragraph of line 260-279, can be trimmed down."*

Author response:
We appreciate the reviewer's suggestions. To facilitate visual comparison among the diets, we modified Fig. 3 (as well as Fig. 1E, 1F, 4, and 6A; see comment #16 below) and present the data as box plots of average time to pupariation. We have also substantially edited and simplified this section of the Results along the lines of the reviewer's suggestions, and focused on interpretation of *Arc1*'s function in GF animals.

5) *"In Figure 5A, what is the basis of the illustrations of "Reduced IIS" and "Over-activated IIS"? Cite at least representative original papers (not reviews), which give evidence for each illustration, in the legend. Again, specify which comparison the authors are discussing, because "smaller cells," "larger cells," "more cells," etc are all confusing."*

Author response:
In order to clarify and simplify these data, and save space for the addition of all of new results, we chose to focus only on trichome density as a measure of wing cell size.

6) *"The authors discuss the data of Figure 5B and eventually stated "Arc1 may also contribute to wing growth through unknown additional mechanisms" (line 378). What mechanism increases the wing cell number of the Arc1 mutant irrespective of Acetobacter sp remains to be solved. This reviewer suspects the possibility that Arc1 is in fact expressed in the wing imaginal disc (see modENCODE Anatomy RNA- seq in FlyBase) and contributes to its tissue growth in a cell-autonomous manner."*

Author response:
We thank the reviewer for bringing the modENCODE expression data to our attention, as this has interesting implications. In lines 481-484 we have mentioned Arc1's reported expression in wing discs and suggest the possibility of its cell-autonomous function contributing towards our trichome density findings.

Points 7-17: Reviewer 3

7) *"1-One criticism is that the experiments were performed at 23˚C rather 25˚C, the optimal developmental temperature for larvae. This lower temperature extends larval development to 7 days, instead of the standard 5. (1 embryonic, plus 4 larval). Additionally, the fact that egg collections/1st instar larvae don't seem to be controlled so that the same number of larvae grow in the vial (10-40 is too much variation, all should around 40-50 ±1, depending on the size of the vials used). Larval density can highly influence developmental and growth rates. Hence it is hard to evaluate with confidence the data presented. All experiments should be performed at 25˚C in a humidified incubator with essentially the exact same number of L1s transferred into vials after short egg collections (usually 4-6 hours)."*

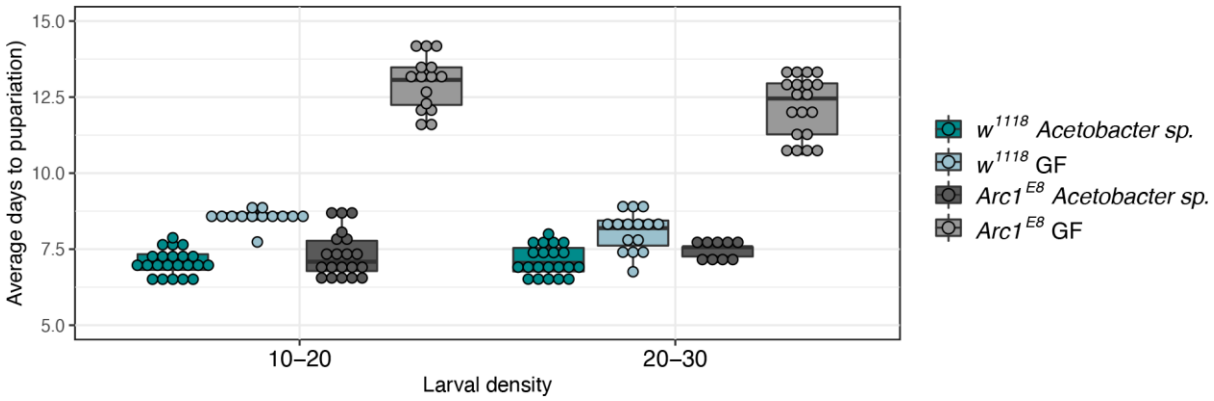
Author response:
We appreciate the reviewer's concerns about our fly husbandry methods, and are well aware of the potentially confounding effects of larval density, temperature, and humidity on growth rate and other phenotypes assayed in our study. Several of the reviewer's specific suggestions are not possible due to technical constraints, but we have made considerable efforts to control for these

variables, and have addressed concerns about temperature and bacterial inoculum concentrations empirically, as described below.

Due to the necessity of maintaining microbiologically sterile and controlled conditions via protocols that are well-established in the *Drosophila* microbiome field (Koyle et al., 2016), it is technically challenging to ensure identical numbers of animals across replicates, as has been noted by others (Troha and Buchon, 2019). As was mentioned in lines 644-647, variable and unpredictable numbers of embryos do not survive the bleach and ethanol treatments used to generate GF cultures; thus transferring identical numbers of embryos to vials does not ensure identical larval densities, and it is not feasible to transfer animals at the L1 stage while maintaining sterility.

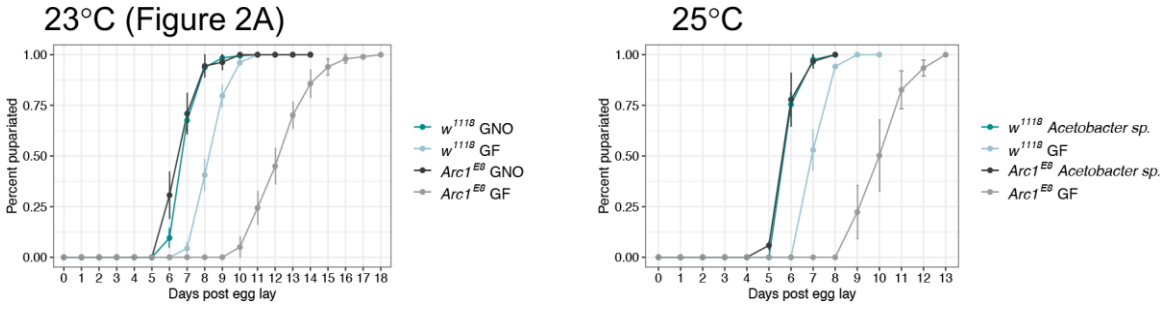
Our lab uses Genessee narrow vials (Cat. No.32-113RLBC), and we have determined that a range of ~10-30 larvae is optimal, as opposed to >40 larvae.

We directly compared developmental rate data for all replicates of the *Acetobacter sp. vs.* GF, w^{1118} *vs.* $Arc1^{E8}$ comparisons at different larval densities, and we found that a range of 10-30 animals produces the same growth rates:



We revised all growth rate experiments from the initial submission (and curated newly added data) by omitting replicates with >30 animals, and repeating experiments as needed for adequate sample sizes. All other experiments used larvae from optimally-crowded vials.

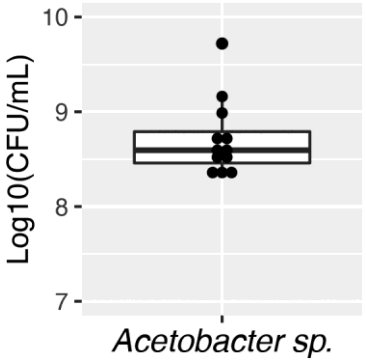
We have also examined *Acetobacter sp.*-associated and GF wild-type and *Arc1* mutant developmental rates at 25¤C, and, while we find that all conditions are accelerated by ~1 day at this temperature compared to 23¤C, the reported trends remain the same:



Therefore, we do not feel that having conducted the entire study at 23¤C presents a cause for concern. Embryo collections were performed for 4-6 hours, and all experiments were conducted in an insect incubator with controlled humidity (70%) and light:dark conditions (12:12 hr), as was indicated in the initial submission and in the revision: Materials and Methods lines 632-633, 639.

8) *"2-40 µL of O/N culture of bacteria doesn't seem to be a precise measure for how much bacteria are supplemented to the diets. Whether dead or alive, bacterial cultures should be grown to the same density, and the same density used each time for each and all experiments."*

Author response:
We quantified bacterial concentration and find that, under our culturing methods, 40µL of overnight culture of *Acetobacter sp.* consistently yields a concentration of ~ $9.06x10^8$ CFU/mL:



Spectrophotometric quantification of heat-killed bacterial samples is likely inaccurate due to incompletely understood cell lysis, an admitted technical limitation of these experiments (we do not state that our data conclusively rule out dead bacterial cells serving as a nutrient source, but rather that the results of these trials do not provide evidence for this function, lines 285-286). Further, it has been established in the *Drosophila* microbiome literature that the association levels of individual bacterial strains is primarily determined by their ability to proliferate on the fly diet, and not influenced by initial inoculum concentration, except at extremes (Koyle et al., 2016; Newell and Douglas, 2014; Storelli et al., 2017). To confirm this, we have repeated the mono-association experiments in Fig. 2F and 2G with a standardized initial inoculum (~10^8 cells) of the four tested bacterial isolates, assaying time to development and quantifying microbial loads in larvae. We found that this had only minor impacts on growth rate in wild-type larvae, while the same trends were observed in *Arc1*^{E8} animals as we reported in the original submission. There was no statistical difference in bacterial loads associated with animals of either genotype (Fig. S4A).

9) *"4-On line 278-9, the authors propose that differences in development observed in the different diets could be due to differences in the levels of bacterial growth on such diets. It is crucial to test this hypothesis and test if indeed bacterial growth is influenced by the different fly diets and if this is an explanation for the changes observed."*

Author response:
We agree with the reviewer that quantification of bacterial abundance on the various yeast-glucose diets is critical to the interpretation of results presented in this section. We have therefore quantified *Acetobacter sp.* abundance in the food substrate for diets on which bacteria-associated larvae develop at differing rates, and find no difference in the levels of live bacterial cells which might correspond to growth rate changes; see Fig. S5, lines 264-266.

10) *"5-What is the result of supplementing Arc1E8 mutants with dead bacteria plus acetic acid?"*

Author response:
We have conducted this experiment and find that rearing GF larvae on 50mM sodium acetate-supplemented sterile food combined with daily addition of heat-killed *Acetobacter sp.* cells does not impact developmental rate compared to GF larvae reared on 50mM NaOAc food without dead bacterial supplementation: Fig. 4B.

11) *"6-Feeding assays were performed for too long. A 3 hour feeding assay saturates guts and any difference in feeding would not be detected. Feeding assays during pre-wandering larval development should be done for 20-30 minutes at the most."*

Author response:
We have repeated food consumption assays with a modified protocol including a shorter, 30 minute feeding period, and find no differences across the four tested experimental conditions: Fig. S4D.

12) *"8-Clonal analysis using wing discs is a better and more accurate approach to conclude what is happening to cellular proliferation and growth. Again, here, growth conditions (temperature, humidity and larval density in vials) have to be more tightly controlled."*

Author response:
We agree that clonal analysis is a more accurate way to assess cell size in an imaginal disc, as it provides an internal control. However, many papers have been published using trichome density as a measure of wing cell size (Brogiolo et al., 2001; Das et al., 2014; Dobens and Dobens, 2013; Rulifson et al., 2002; Shin et al., 2011). Because of the precedent in the literature, the fact that this is a relatively minor point in our manuscript, and the significant amount of effort that would be involved in such analysis, we did not repeat these experiments using clonal analysis. We hope that the reviewer will agree that this is not a significant concern.

13) *"10-Conclusions drawn in the discussion have to be toned down to more accurately represent the results"*
14) *"a)The link between Arc1 and IIS signaling is not novel. Mattaliano et al, observed a link between Arc1-expressing cells and IPCs and Mosher et al, proposed that Arc1 regulation is downstream of Ilps. The present findings should be better described in the context of these prior findings throughout the paper and especially in the Discussion."*

Author response:
We have substantially modified the Discussion in light of our new data, and made efforts to more directly and thoroughly contextualize our findings in relation to Mattaliano et al., 2007 and Mosher et al., 2015 throughout the manuscript.

15) *"b)Line 285-7: enhanced starvation survival could be solely due to the observed increase in fat levels for the mutants, please also mention this possibility."*

Author response:
We agree with the reviewer that the starvation resistance of *Arc1* mutant adults is likely at least a partial consequence of increased body fat levels observed during the larval stages, and have stated this possibility in lines 332-333.

16) *"c)Figures 3 and 4 are too small to read as presented."*

Author response:
We have modified the figures as indicated above to improve legibility and facilitate comparison among the different treatments.

17) *"d)Add reference to figure 5E on line 382."*

Author response:
The indicated text and figures have been modified substantially.

Second decision letter

MS ID#: DEVELOP/2020/195222

MS TITLE: Arc1 and the microbiota together modulate growth and metabolic traits in Drosophila

AUTHORS: Scott A. Keith, Cassandra Bishop, Samantha Fallacaro, and Brooke M. McCartney

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Reviewer 1

Advance summary and potential significance to field

The revised work has adressed my initial concerns, I am supporting publication of the MS as it is. F.Leulier

Comments for the author

The revised work has adressed my initial concerns, I am supporting publication of the MS as it is. F.Leulier

Reviewer 2

Advance summary and potential significance to field

The manuscript of Keith et al. describes how Drosophila-associated bacteria species contribute to larval growth on several distinct diets. Two of the authors'
interesting findings were that (1) Arc1 mutant larvae exhibit a marked delay in pupariation, compared to wild-type animals, under a germ-free (GF) condition (the phenotype in developmental timing in GF) and (2) transplantation with Acetobacter sp. alone totally cancels this phenotype. Arc1 is an evolutionally conserved gene and plays a pivotal role in regulation of synaptic plasticity. To unravel mechanisms underlying the timing phenotype and the effect of Acetobacter sp., authors performed three-way analysis: the genotype of larvae (wild-type or Arc1 mutant), GF or gnotobiotic/GNO, and the diets.

Comments for the author

Due to the authors' substantial amounts of additional data and the extensive textual revision, the original manuscript has been reincarnated as an almost new manuscript. This reviewer appreciates with an additional conclusion: "Notably we found that molecular readouts of IIS function at multiple points in the pathway, and in multiple tissues, were altered in Arc1 mutants, though in both microbiota-dependent and -independent ways, and not consistent with simple IIS over- or under-activation (line 526-529)."

There is still a major reason why the revised manuscript is not necessarily easy to read. In every graph except for that in Figure 2F or G, what comes leftmost is the data of mono-association of with a single Acetobacter sp. isolate. It would be straightforward to start with the data of germ-free wild-type larvae in the leftmost position and show the effect of mono-association in the second from left. However, this reviewer suggests only minimal textual revisions at this stage.

1. Line 244-246 "The developmental rate of wild-type GF larvae is particularly sensitive to dietary yeast, the primary source of ingested amino acids, and also to food carbohydrate content (Shin et al., 2011; Storelli et al., 2011; Wong et al., 2014)." As pointed out in the original review, yeast is a

rich source of many nutrients, including unknown but critical to larval growth (see Figure 5a in Piper et al. 2014). The revised sentence would be “The developmental rate of wild-type GF larvae is particularly sensitive to dietary yeast, the primary source of ingested amino acids and many other nutrients (Shin et al., 2011;
Storelli et al., 2011; Wong et al.,2014 Piper et al. 2014).”
Piper et al. Nature Methods (2014). A holidic medium for Drosophila melanogaster.

2. Line of 408-409 “We found that Ilp3 and Ilp5, but not Ilp2, transcripts were reduced ~2 fold in the brains of feeding GF Arc1[E8] larvae compared to wild-
type larvae (Fig. 7A). By contrast, Ilp2 and Ilp5 protein levels were increased in Acetobacter sp.-associated Arc1 mutant brains (Fig. 7B).” The authors mixed up the multiple genes, the genotypes and germ-free vs. association. What can be stated here may be “Irrespective of the changes of the transcript amounts, both Ilp2 and Ilp5 protein levels are increased in Acetobacter sp.-associated Arc1 mutant brains compared to germ-free Arc1 mutant brains.”

3. Line 435-441. Specify gene symbols “sro” and “shd” of “Shroud” and “shade,” respectively.

4. Line 580. What is the feature of the fly stock Top Banana? Any reference?

Reviewer 3

Advance summary and potential significance to field

This manuscript describes a new identified role for Arc1 responding to microbiota changes in the gut to coordinate growth and development of the fly. The authors get deeper into the mechanism by showing supporting evidence that Arc1 is required for insulin as well as 20E signaling. This is the first time Arc1 has been shown to be involved in microbiota responses.

Comments for the author

This manuscript describes a newly identified role for Arc1 responding to microbiota changes in the gut to coordinate growth and development of the fly. The authors get deeper into the mechanism by showing supporting evidence that Arc1 is required for insulin as well as 20E signaling. This is the first time Arc1 has been shown to be involved in microbiota responses.
In this revised version of the manuscript, Keith et al., have thoroughly addressed all my concerns. It was a joy to read this much improved revised submission as it scores at the top of my list as one of the best addressed responses to reviewers I have read. Well done, authors!

A few minor issues to revise:
1. Please ensure lines in the graphs in figures 2A, B, 5E and 6 B, C G are thicker for better visualization.

2. Please ensure the genotypes in the Methods includes the w backgrounds, when applicable. Most of these lines are in a w background, and since w were also (appropriately) used as controls, it is important to emphasize this fact (for example w*; Arc1 ems 13,…).

3. On line 373, please also reference the Mattaliano manuscript, as they showed this link for the first time.

Also important to note (in the Discussion perhaps) that their conclusion was that Arc1 cells fully overlap with IPC cells, whereas you find only a partial overlap with this cells in addition to the neighboring region.

4. It is unclear what the a b c … in the figures mean. Please add a description to the legend and also in the methods in the statistical descriptions (I think these may represent different intervals for p-values?).

5. In methods, please add catalog numbers to the reagents. This is especially important for those used in the food and dietary experiments (cornmeal, yeast, propionic acid, glucose, glacial acetic acid, sodium acetate, etc), and in general to all other reagents (such as antibodies,…) and/or kits used.

6. Please specify the concentration of Red#40 dye used in the yeast paste for the feeding assays.

7. Please better describe the density of the Acetobacter sp. cultures used for the heat-killed experiments;
"overnight" can represent a range of times and, unless the culture has saturated long before, can lead to a variety of different densities.

8. Please provide the full software reference for Fiji.

Second revision

Author response to reviewers' comments

We thank the reviewers for their positive evaluation of our manuscript revisions. We have completed each of the requested text and figure modifications listed below, which improve clarity and methods transparency.

Reviewer 2 comments

"1. Line 244-246 "The developmental rate of wild-type GF larvae is particularly sensitive to dietary yeast, the primary source of ingested amino acids, and also to food carbohydrate content (Shin et al., 2011; Storelli et al., 2011; Wong et al., 2014)." As pointed out in the original review, yeast is a rich source of many nutrients, including unknown but critical to larval growth (see Figure 5a in Piper et al. 2014). The revised sentence would be "The developmental rate of wild-type GF larvae is particularly sensitive to dietary yeast, the primary source of ingested amino acids and many other nutrients (Shin et al., 2011; Storelli et al., 2011; Wong et al.,2014 Piper et al. 2014)."Piper et al. Nature Methods (2014). A holidic medium for Drosophila melanogaster."

Author response:
We have edited this sentence as requested, and included the citation of Piper et al., 2014, *Nature Methods*.

"2. Line of 408-409 "We found that Ilp3 and Ilp5, but not Ilp2, transcripts were reduced ~2 fold in the brains of feeding GF Arc1[E8] larvae compared to wild-type larvae (Fig. 7A). By contrast, Ilp2 and Ilp5 protein levels were increased in Acetobacter sp.-associated Arc1 mutant brains (Fig. 7B)." The authors mixed up the multiple genes, the genotypes and germ-free vs. association. What can be stated here may be "Irrespective of the changes of the transcript amounts, both Ilp2 and Ilp5 protein levels are increased in Acetobacter sp.-associated Arc1 mutant brains compared to germ-free Arc1 mutant brains.""

Author response:
In accordance with this suggestion, we have edited the text (lines 407-411) to read as follows: "We found that *Ilp3* and *Ilp5*, but not *Ilp2*, transcripts were reduced ~2 fold in the brains of feeding GF *Arc1*^{E8} larvae compared to wild-type *Acetobacter sp.* associated larvae (Fig. 7A). Irrespective of these transcript level changes, both Ilp2 and Ilp5 protein levels were increased in *Acetobacter sp.*-associated *Arc1* mutant brains compared to GF Arc1 mutant brains (Fig. 7B)." We feel these statements accurately describe the data represented in these figure panels.

"3. Line 435-441. Specify gene symbols "sro" and "shd" of "Shroud" and "shade," respectively."

Author response:
We have specified the gene symbols used in Figure 7G in the main text as requested, lines 435, 439.

"4. Line 580. What is the feature of the fly stock Top Banana? Any reference?"

Author response:
The Top Banana stock is derived from flies that were wild-caught in Seattle, Washington in 2013 by Dr. Michael Dickinson's lab at CalTech, as noted in Giraldo et al., 2018, *Current Biology*. We have cited this reference and described the provenance of the Top Banana stock in the "*Drosophila* stocks and diets" section of the Materials and Methods, lines 580 582.

Reviewer 3 comments

"1. Please ensure lines in the graphs in figures 2A, B, 5E and 6 B, C G are thicker for better visualization."

Author response:
As requested, we have increased the line thickness for all developmental rate and survival curves in both main text figures (Fig. 2A,B, Fig. 5E, Fig. 6B,C,G) and supplemental figures (Fig. S2A-C,E, Fig. S6, Fig. S8, Fig. S9).

"2. Please ensure the genotypes in the Methods includes the w backgrounds, when applicable. Most of these lines are in a w background, and since w were also (appropriately) used as controls, it is important to emphasize this fact (for example w; Arc1 ems 13,…)."*

Author response:
We have updated the "*Drosophila* stocks and diets" section of the Materials and Methods to specify *w* or *yw* genetic backgrounds for all mutant and transgenic lines utilized in our study.

"3. On line 373, please also reference the Mattaliano manuscript, as they showed this link for the first time. Also important to note (in the Discussion perhaps) that their conclusion was that Arc1 cells fully overlap with IPC cells, whereas you find only a partial overlap with this cells in addition to the neighboring region."

Author response:
We have added the following sentence to this section (lines 374-375), referencing the indicated publication: "In the adult brain, Arc1 staining fully co-localized with the IPCs (Mattaliano et al., 2007)." Importantly, Mattaliano et al. (2007) present data showing Arc1 co-localization with: 1) IPCs in adult brains (anti-Arc1, *Ilp2>GFP*, Figure 6A1-A3) and 2) AKH-positive cells in the larval ring gland (anti-Arc1, *Akh>GFP*, Figure 6B1-B3). Our study is the first to document Arc1-Ilp co-localization in the larval brain.

"4.It is unclear what the a b c … in the figures mean. Please add a description to the legend and also in the methods in the statistical descriptions (I think these may represent different intervals for p-values?)."

Author response:
Lowercase letters in the figures represent statistical results of post hoc, pairwise comparisons from ANOVA or Kruskal-Wallis tests. We include the statement "Conditions that share a letter are not statistically different from one another" in the legends accompanying each figure in which we use this notation. Statistical tests used for each panel are indicated in the figure legends as well. As requested, we have also added an explanation of the letter-based notation to the "Statistical analysis" section of the Materials and Methods, lines 793-795.

"5. In methods, please add catalog numbers to the reagents. This is especially important for those used in the food and dietary experiments (cornmeal, yeast, propionic acid, glucose, glacial acetic acid, sodium acetate, etc), and in general to all other reagents (such as antibodies,…) and/or kits used."

Author response:
As requested, we have added catalog numbers for all reagents indicated in the Materials and Methods. Exceptions are cornmeal and molasses utilized in our lab's routine fly diet, which our Department does not obtain from scientific vendors. We have added additional identifying information for these reagents (lines 593-594).

"6. Please specify the concentration of Red#40 dye used in the yeast paste for the feeding assays."

Author response:
For feeding assays, we utilized a 2:1 yeast paste mixture consisting of 0.5% aqueous solution FD&C Red #40 dye (Ward's Science; Cat. No. 470301-004) and active dry yeast. We have specified these proportions and concentrations in lines 666-667.

"7. Please better describe the density of the Acetobacter sp. cultures used for the heat- killed experiments; "overnight" can represent a range of times and, unless the culture has saturated long before, can lead to a variety of different densities."

Author response:
To clarify these methods as requested, we have specified that all overnight cultures were grown for ~16-18 h ("Generation of germ free and gnotobiotic cultures" line 628). In line 686, we note that *Acetobacter sp.* cultures grown in this time frame range in cell density from ~$2.5x10^8$-10^9 CFU/mL, which we quantified and reported in the previous "Response to reviewer comments" document (see data in Author response to Point #8).

"8. Please provide the full software reference for Fiji."

Author response:
In lines 659-660 we have provided the original publication reference for Fiji (ImageJ):
Schindelin, J.,Arganda Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: An open-source platform for biological image analysis. *Nat. Methods*. 9, 676-682.

Third decision letter

MS ID#: DEVELOP/2020/195222

MS TITLE: Arc1 and the microbiota together modulate growth and metabolic traits in Drosophila

AUTHORS: Scott A. Keith, Cassandra Bishop, Samantha Fallacaro, and Brooke M. McCartney
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.