Additional modifications have been highlighted in blue in the manuscript.

Reviewer 2 :

>>I believe the authors did not get the point of my major concern. That being, that given what we know (from published work) of the nature and association of the Rhodnius-Rhodococcus symbiosis, it is reasonable to believe that additional bacterial species with the capacity to supplementnutritional deficiencies can have an easier path to being maintained vs. what is observed insymbiotic associations which display more intimate characteristics. I do not believe the mode of transmission is an explanation for the lack of genome reduction or other typical characteristics of obligate nutritional symbionts. For example, you have Ishikawaella capsulata, that displays extensive genome reduction and an A+T-biased genome while being extracellular and being inherited through symbiont capsules deposited on the external surface of the eggs. What is most important is whether the vertical transmission is "leaky", in a sense of the possibility of closely related bacteria recombining with the "true" symbiont or even taking over the infection of the developing host. Now, the prevalence of Rhodococcus is certainly good evidence for some sort of true symbiotic role, which in my opinion certainly includes B-vitamin provision. However, other roles could also fall onto this symbiont, given its extensive gene set. Also, one can imagine that the finely-tuned regulation on gene expression that exist, in say Wigglesworthia-Glossina (https://doi.org/10.1128/AEM.02052-12), might not exist in the Rhodnius-Rhodococcus symbiosis, and this is what might leave the door opened for other symbionts, such as Wolbachia or others, to overtake or supplement some of these essential functions. So, my initial comment and concern still stands. I do not believe there is enough evidence to “speculate that R. rhodnii and Wolbachia compose an ancient and dual association of co-symbionts, as seen in many other hemipteran”. Rather, I believe what the author's data show is the potential for a nutritional complementation/supplementation by Wolbachia. Therefore, I strongly the aforementioned "speculation" need to be remove and/or nuanced in a similar way as I suggest.<<

We have removed the problematic sentence and moderate the statement in the introduction (L59) and in the conclusion (L671)

>> Here I would like to start by stressing one of the points raised by Reviewer 1 on contamination. It is a good starting sign that indeed the authors do not find contamination with nematode nuclear DNA. As I believe this is an important point and due to the lack of specific FISH microscopy analyses, that the authors also make sure that no nematode mitochondria are recovered. The reasoning behind this is that, despite these contaminations generally being low (or of low coverage unless high infection is present), the mitochondria, as do the endosymbionts, tend to have much higher coverage than nuclear data

(sometimes several hundred times higher). So, despite not finding many nematode hits, one can find even complete mitochondria (I seldom whole mitochondrial genomes of parasitoid wasps in my aphid data or nematode in my leech data while no nuclear DNA is found of these contaminations), which would then raise the question about the origin of the Wolbachia contigs.

To go deeper and to be sure that no Nematode contamination is present, we used the complete *C. elegans* mtDNA genome as a seeds to search with BLASTN for Nematod DNA : we failed to obtain any hits for all the samples infected with Wolbachia. Even with the protein C. elegans CoxA sequence and TBLASN searches we only retrieved CoxA *Rhodnius* sequences and no Nematode sequences. The absence of mtDNA from Nematod in our samples strongly suggest that there is no contamination, strengthening the blobplot analysis presented in our initial response to R1.

>>I would reiterate, synteny cannot be evaluated with such fragmented genomes. For example, in a genome with an N50 of 2 to 7Kbp, one cannot evaluate synteny beyond a string of about 2-7 genes
on average. In addition, I would remind the authors that, as a general rule, these mobile-element-rich genomes tend to break synteny in repeat elements, such as those that break an assembly of anorganism such as these Wolbachia done with only short reads. As examples of works analysing this pehnomenon see https://doi.org/10.1101/2022.05.31.494226
and
https://doi.org/10.1093/gbe/evu133 <<

We have removed this part of the figure 3 and deleted all the mentions of the synteny in the manuscript.

>> I do not believe the statement that Chimeric sequences would have been generated at the same ratio due to the use of the same sequencing (and I imagine library prep) technology. The generation of Chimeric sequences depend on many factors, one of which would be the DNA ratios of the different genomic molecules in there. For example, if the amount of Wolbachia relative to the host DNA is variable, one can expect different amount and type chimeric sequences to be generated. Similar expectations would be true if the genomes vary, which judging from Fig 2., this seems to be true for Wolbachia. These chimeric regions would of course cause breaks in the assembly, and would thus leave them in contig ends. Chimeric regions can especially be generated with low coverage data, which is something to be especially aware of. So, I would still suggest that the claim, especially for putative HGTs located close to contig ends, to be presented with the nuance that is required and highlighting the limitations of the data in hand. <<

Honestly, we don’t understand why chimeric sequences would be generated with such high ratio and systematically for all genomes, including those for

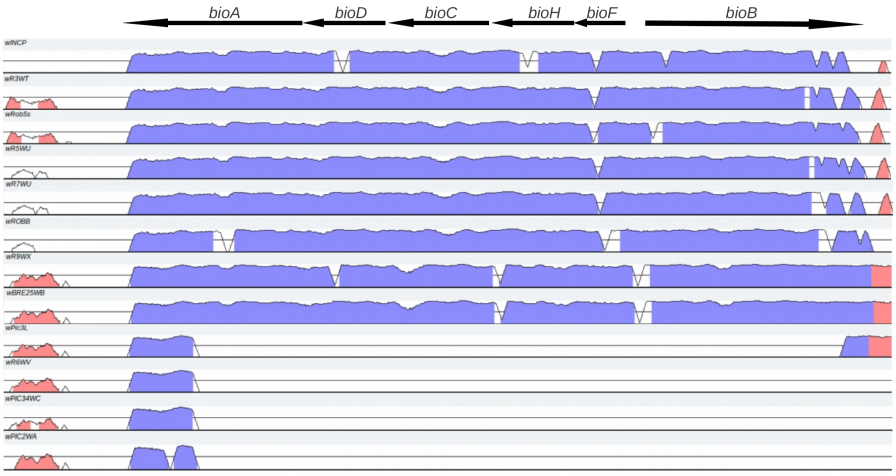
which free *Wolbachia* have not be evidenced by genome sequencing and PCR. However, we have added a sentence to indicates this possibility.

>>OK, but then, if no missing data was retained in the alignments, why thinking the clustering is
erroneous? I would think that the case of RobQ suggests that Wolbachia can also horizontally get
transferred and likely even replace that present in the receiving lineage, right? I would also say that
the grouping of RobQ is not "aberrant", but unexpected given the a priori assumptions. <<

Yes you right, now we realize that the word “aberrant” is a bad translation from the same french word that have a different meaning, the right word is “unexpected”.

>>When assembling a genome from such a metagenome, the coverage, especially of such a lowly abundant genome in the mix, is not (at all) normally distributed, which means that while some regions might be 10x, some might be closer to cero. Thus, this is why some parts can be better and more universally represented than others. If coverage is sufficiently high, one usually has no such problem (and can easily extract all interconnected scaffolds/contigs from an assembly graph and thus insure to a high certainty completeness). For the lowly covered genomes, I would think this is
an important issue. So, I'm not saying all (or even most) of the gene losses that you observed are due to this, but it is definitely an important caveat to mention and be taken into account to nuance conclusions based on your presented data. <<

It’s not true that the genome coverage of our Wolbachia genome is low :
7 have genomic coverage >200x. In fact only 3 genomes have coverage <50x. See Table 3. It’s true that the assembly are fragmented due to the of use of small illumina reads but the the coverage is largely sufficient to assemble genomes with comparable size of wCle and to perform presence/absence analysis of peculiar genes. In the *Wolbachia* phylogeny resulting from the alignment of the dataset of Commandatore et al. paper, we were able to find for all of the samples 80 genes on a total of 90. Without the (incomplete) RobQ sample, 89 are present in all of the samples. Some are fragmented in multiple contigs but they are still identifiable.
If we take as an additionnal example the loss of the biotin operon in the *pictipes* lineage, the genomic deletion is located exactly at the same position in the four regions as indicated in supplementary Fig 2 :



This pattern is incompatible with artificial random deletions generated by insufficient coverage of some regions. This is particularly true for such large operons that span several kilobases. This correspond to real genomic deletions in the ancestor of the *pictipes* lineage. For all of these reasons we are really confident that our presence/absence analyzes are reliable.

>> I reiterate, the close phlyogenetic relation and genomic similaritues between two Wolbachia strains present in two distantly related hosts with different biology is not evidence of a nutritionalmutualism as a shared phenotype. Therefore, I do not believe that the aforementioned data provides any support whatsoever to the nature of the Wolbachia symbiotic association. <<

We have moderate the statement in the discussion section (L621). At this stage, it becomes difficult to be more cautious...