nature portfolio

Peer Review File

Spatial constraints drive amylosome-mediated resistant starch degradation by Ruminococcus bromii in the human colon

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Wimmer et al. have investigated the assembly and activity of the Ruminococcus bromii amylosome in an ambitious bid to understand how this keystone organism achieves the efficient digestion and utilization of resistant starch. While the experiments have generally been well conducted, there would seem to be additional work that is needed in order to complete this story. The proteomics of the secretome and cell associated proteins offer a critical and interesting look at what is different between these two locations, but I think ultimately need to be complimented by transcriptomics to understand how much of the dynamics is via transcriptional regulation and how much is by other mechanisms such as proteolysis that is mentioned in the paper. For instance it is unclear how the organism is able to differentiate between the resistant starch and the soluble starch that will contain all of the same components and linkages even if they are at a different ratio. Perhaps transcriptomics with these substrates and potential inducer molecules such as maltose could shed light on this as the differences between the RS and soluble starch are primarily physical, rather than chemical. Besides this there are other issues, some minor, some potentially major that need to be addressed and are outlined below.

Introduction
Line 59 – this should be updated to say that only a few bacterial species have been identified as RS degraders within the human gut microbiota. There are others in other environments.

There should likely be some note that for raw potato starch, B. adolescentis is generally found to be the most responsive. E.g. see https://doi.org/10.1016/j.nutres.2020.02.009

Line 129 probably should specifically note that it cannot use glucose

Amylosome structure/composition
The Figure 1 legend notes that the amylosomes are linked to the cell wall through ‘elongated linkers’. What protein is serving as the linker between the bacterial cell and the amylosomes? Presumably this would be Sca2 and/or Sca5. Are these distances compatible with the size of these proteins?

What mechanism could maintain a consistent ratio within the cell-free amylosomes, while allowing the composition of the cell-associated amylosomes to vary radically? This never really seems to be discussed.

Line 238-241 is a bit worrying. If the components that are proposed to be essentially what defines a cell-associated amylosome are not being detected (even if they are in general confirmed to be present) the confidence in what is being detected being an unbiased representation would seem to be called into question.

The pullulan induced amylosome not including any of the pullulanases seems odd. This is clearly suboptimal for the bacteria, so what is driving this?

General
Line 198-201 I’m not sure I understand the link between these proteins and human health promotion.

Line 269-271 No mention of amylopectin when discussing the structure of starch? Even high amylose varieties are 15% or (usually) more amylopectin.

Figure 6 – I'm not really sure what value this figure is providing. Figure S15 seems to be a much more useful representation of the findings, maybe just focused on the resistant starch part. Possibly could be combined with 6b. 6a, seems better suited to being part of figure 1 as part of the explanation of the results seen there or as a supplemental that is discussed in conjunction with figure 1.

Enzyme structure and activity
Line 316-318 So the suggestion is that Amy4 is in fact a reducing end specific glucosidase? To make this claim would require additional biochemical characterization. Looking at activity towards a range of substrates (including those with a blocked reducing end) and ensuring that glucose is the main product released would be necessary.

I'm also not sure what the point of comparing Amy4 (amylase) and Amy10 (pullulanase) is here. There are many other amylase and pullulanase structures that would need to be included to conduct such an analysis properly. It also doesn't really seem to be relevant as it is already clear that they have different substrate preferences. A comparison between the Amy10 and Amy12 structures would seem to be more relevant to possibly identify differences that could lead to different substrate/product profiles of the two enzymes.

Figure S8, which provides the information for Figure 3b and the interpretation of the activity of Amy16. I'm not really convinced of much of anything by those TLC figures. To demonstrate alpha1,6 activity you would specifically need to see maltotriose production, not just an indication of activity. The open active site of Amy16 is reminiscent of the B. thetaiotaomicron SusG enzyme, which has activity against pullulan, but functions as a neopullulanase, cleaving the alpha1,4 bonds within pullulan. The open active site architecture allows it to accommodate branch points close to the alpha1,4 bond that it cleaves, but does not allow direct hydrolysis of alpha1,6 bonds. Additional characterization is needed to identify the products from pullulan hydrolysis (e.g. panose = neopullulanase, maltotriose = pullulanase). This can be done by TLC, with better standards and more care, but HPAEC-PAD would be the gold standard

Figure S10 and S11 both appear to show all of the details for Amy12 purification and structure determination, whereas the legend for S10 suggests that ii should contain the details for Amy10.

Substrates - How were the substrates prepared? Was the RS treated the same as for the growth experiments? Any heat treatment? For RS it is customary to subject them to mock upper intestinal digestion (eg. INFOGEST) to mimic what substrates the bacteria would actually encounter in the large intestine. Additionally to make any conclusions about activity towards RS, a number of different RS substrates should be tested (they vary quite dramatically in physical properties that are highly likely to influence enzymatic activity).

Media and growth
how were the media prepared, were they all autoclaved? The Hi-Maize starches are relatively high gelatinizing, but from what I can find H-Maize 958 has a peak gelatinization temperature of just under 100 Celsius. So if subjected to a standard autoclave procedure it would be gelatinized. So more details are needed.

The M2 media is undefined, containing for instance clarified rumen fluid and yeast extract. Could these components contain factors (e.g. carbohydrates) that are activating the expression of some of the amylosome components, masking differences between the carb sources used in this study?

Reviewer #2

(Remarks to the Author)
This study presents a comprehensive and methodologically robust investigation into the architecture and functional dynamics of the amylosome complex in Ruminococcus bromii, a key microbial species involved in resistant starch (RS) degradation in the human gut. The authors employ a multilayered approach—integrating cryo-electron tomography, proteomics, and biochemical assays—to elucidate how structural and regulatory mechanisms govern enzymatic synergy and substrate degradation. The major finding of their work are its demonstration that amylosome function is modulated at two distinct levels: (1) spatial organization that enforces proximity among enzymes, and (2) condition-dependent expression shifts that alter enzyme composition. Notably, the study identifies Amy4 and Amy16 as dominant components under RS-rich conditions, accounting for 60% of the amylosome's enzymatic profile. Structural and functional analyses further reveal a synergistic interaction between these enzymes, enhancing RS breakdown and highlighting the organism's adaptive capacity to dietary fiber availability. Overall, this article is very well written and presented and makes a significant contribution to our understanding of microbial fiber degradation and its implications for colonic fermentation and host-microbiome interactions. The integrative methodology and mechanistic insights offer a valuable framework for future studies on gut microbial ecology and metabolic engineering. I have the following comments for consideration:

In the section detailing the enzyme structure there are many predictions made as to the specificity of the CAZymes based on the size and shape of the catalytic binding site/cleft. When reading this the thought arises to actually test these substrates against Amy4 and Amy16, which of course they authors do in the following section. Is there not a way to slightly modify the text to at a minimum make this connection between the two bodies of work obvious to the reader when discussing the

structures. It is also not clear if validating biochem assays were performed to confirm the predictions of linear vs branched substrates for say Amy10, 12 vs Amy4? Finally, given the structural differences of say Amy4 and 16, yet their tight and essential association I am curious if the authors have any hypothesis as to how they interact and coordination hydrolysis of a RS substrate?

When reading the manuscript I was curious if the authors had any insight into the key molecular triggers when a given substrate is exposed to the cell. In gram- PULS there is very often a two-component systems (TCSs), which are signal transduction pathways that allow cells to adapt to environmental changes. The triggers are often the corresponding disaccharide from the target substrate. When reading the final discussion/conclusions I see the author do in fact address this and state it is unknown. Nonetheless, I do wonder if there were any indicators in the gene organisation and/or the proteomes that indicate how the cells differentiate and respond to the changing substrates?

On line 198 the authors state: “These included an annotated fructose-1,6-bisphosphatase, part of the gluconeogenesis pathway, and two annotated ATPases, potentially linking RS degradation to human health promotion through cross-feeding and the release of beneficial metabolites.” It is not clear to me how the idenfication of a common gluconeogenesis enzyme and ATPases links RS degradation to human health promotion. EIther some more context needed here or some adjustment of the sentence.

The authors use proteome abundances and % to great detail to explain the amylosome, a short clear concise sentence how they did this in the main text could be beneficial to the reader (it is detailed clearly in the methods)

Reviewer #3

(Remarks to the Author)
This manuscript presents an integrative study of the amylosome in Ruminococcus bromii, a multi-enzyme complex involved in resistant starch (RS) degradation. Combining cryo-electron tomography, cryo-EM, proteomics, and biochemical assays, the authors aim to elucidate the structural and functional basis of RS metabolism. While the study generates extensive datasets, the central mechanistic narrative, focusing on spatial synergy and functional complementarity, remains speculative and insufficiently supported by the current analyses. Better data integration, further experimental validation, and more restrained conclusions would substantially improve the manuscript.

Major Comments:

1. The central hypothesis remains descriptive:
The authors claim that “spatial constraints” within the amylosome drive enzyme synergy—is conceptually intriguing but remains speculative due to a lack of mechanistic evidence. While cryo-ET reveals extracellular densities and proteomics shows Amy4 and Amy16 enrichment on RS, these observations do not demonstrate direct spatial proximity. There is no experimental validation of physical interactions between enzymes on the native amylosome. SEC suggests that the complex forms, but further validation is necessary— e.g., freezing SEC fractions on EM grids to see if the structure of the complex can be determined, or using crosslinking-MS, proximity labeling. Otherwise, you may revise the narrative to reflect the current limitations in mechanistic insight.

Synergy between Amy4 and Amy16 is shown via TLC and BCA assay, but no enzyme kinetics are provided. Also, have you confirmed that the substrate concentration in the BCA assay falls within the linear detection range? If not, this may lead to misinterpretation of the observed synergistic effect.

2.Cryo-ET lacks in-depth analysis:
The tomographic data are interesting but not fully mined. Only 21 tomograms are analyzed in detail. The blob-picking strategy is vaguely described and does not clearly define what is being measured. The STA approach is only shown for a ribosome control but fails to extract meaningful information from the amylosome layer, likely due to heterogeneity. This point is not critically discussed. Include quantitative segmentation or mapping of amylosome density across the cell perimeter to support claims of architectural regularity. Consider focused classification attempts (e.g., using EMAN2 or Warp/M) on amylosome-associated densities—even unsuccessful attempts would help clarify limitations.

3. Proteomics dataset is extensive but underinterpreted:
The proteomic dataset is extensive but lacks rigorous interpretation. The criteria for “RS-specific” proteins are inconsistent: sometimes defined by presence/absence (Jaccard), other times by intensity ranks. Several major observations (e.g., Amy16 increase) are reported descriptively but not statistically validated at the protein level (only at the core proteome level). The PCA and ANOVA are presented, but not linked to biological conclusions. Apply differential expression analysis (e.g., LIMMA or DEqMS) to identify significantly upregulated proteins across RS vs. other substrates. Clarify how rankings and fold changes were calculated. A volcano plot or heatmap with adjusted p-values would add interpretive rigor.

Minor comments:
•Several figures (e.g., Fig. 2, Fig. S5) are dense and lack adequate legends or in-text guidance. Subpanels are not cited consistently, and it's hard to follow which datasets support which claims. Simplify main figures, provide better in-text figure references (e.g., “Fig. 2c, right panel”), and summarize key quantitative findings in tables.

•While loop differences and pocket accessibility offer insight, they do not fully account for dynamic substrate binding. The conclusion about substrate promiscuity versus specificity might be premature without substrate-bound structures or molecular dynamics simulations.

•Have you mutated the C-terminal loop (e.g., H383/W384 in Amy4) to test its role in substrate specificity?

•A more detailed comparison of the active sites of Amy4 and Amy16 is needed to clarify how structural differences contribute to their distinct substrate preferences. This includes analyzing specific loop architectures and residue-level interactions that may affect active site accessibility.

Reviewer #4

(Remarks to the Author)
I co-reviewed the paper with Reviewer #3. I thank you for the opportunity. The paper is good and interesting but it needs some work and polishing from the authors.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed all of my concerns. I will only leave the additional small comment, that while the trascriptional profile is different between the soluble starch and resistant starch, the fact that none of the amylosome components show up in this analysis, suggest that the amylosome compositional differences are not controlled by transcriptional regulation. However, elucidating the full mechanism by which they are controlled, is likely beyond the scope of this manuscript.

Reviewer #3

(Remarks to the Author)
The new data and clarifications have substantially improved the manuscript. The addition of statistical and structural analyses, biochemical results, and a clear acknowledgment of the limitations of cryo-ET and in situ data strengthens the work. The synergistic effect of Amy4 and Amy16 is convincingly demonstrated.
I have only minor suggestions:
-Proteomics data show strong enrichment of Amy9 on RS, yet biochemical assays indicate little activity against RS. While the manuscript clarifies differences between autoclaved and non-autoclaved starch, the precise role of Amy9 in the amylosome remains unclear. Based on the predicted Amy9 structure or existing literature, the authors could briefly speculate on its potential function and why it does not show a synergistic effect, or simply acknowledge this as an open question for future research.
-You note that the cell-wall anchored scaffoldins Sca2 and Sca5 were not consistently detected in the proteomics data. This is an important detail. You could make the connection between the transcriptomics and proteomics results more clear, perhaps by highlighting this as a limitation of the current study to provide a complete picture of amylosome regulation.
There is a typo in Figure 2 legend: “Crbon” → “Carbon.”

Reviewer #4

(Remarks to the Author)

Reviewer #1 (Remarks to the Author):

Wimmer et al. have investigated the assembly and activity of the Ruminococcus bromii amylosome in an ambitious bid to understand how this keystone organism achieves the efficient digestion and utilization of resistant starch. While the experiments have generally been well conducted, there would seem to be additional work that is needed in order to complete this story. The proteomics of the secretome and cell associated proteins offer a critical and interesting look at what is different between these two locations, but I think ultimately need to be complimented by transcriptomics to understand how much of the dynamics is via transcriptional regulation and how much is by other mechanisms such as proteolysis that is mentioned in the paper. For instance it is unclear how the organism is able to differentiate between the resistant starch and the soluble starch that will contain all of the same components and linkages even if they are at a different ratio. Perhaps transcriptomics with these substrates and potential inducer molecules such as maltose could shed light on this as the differences between the RS and soluble starch are primarily physical, rather than chemical. Besides this there are other issues, some minor, some potentially major that need to be addressed and are outlined below.

We thank the reviewer for this insightful comment and appreciation of our work. Indeed, integrating transcriptomics with proteomics could provide additional valuable information. To this end we have used a transcriptomic dataset of *R. bromii* grown on starch and resistant starch that was previously published (Crost et al., 2018). By re-analyzing this dataset using DESeq2 and aligning it to *R. bromii* L2-63 genome we uncovered a specific and significant transcriptional response of the bacterium to the respective carbon sources (ANOSIM R=0.778, p-value 0.1). This suggests that soluble and resistant starches induce differential transcriptional responses due to their physical properties, possibly based on differences in accessibility and solubility.

We discovered a set of 26 genes (Fig. S18, Table S6) that are significantly upregulated when the bacterium was grown on soluble starch (compared to resistant starch). Importantly, we observed the upregulation of several genes directly involved in carbohydrate metabolism. These include a phosphotransferase which is potentially linked to sugar uptake, and phosphorylation—a key step in the phosphotransferase system (PTS) for carbohydrate transport—as well as a pentose-5-phosphate-3-epimerase, which participates in the pentose phosphate pathway essential for processing sugars into metabolic intermediates. Additionally, we identified an upregulation of an NAD(P)-dependent alcohol dehydrogenase, likely involved in downstream fermentation of sugar-derived products, and a ketose-bisphosphate aldolase, typically associated with glycolysis and gluconeogenesis. These upregulated proteins suggest

an activation of central carbohydrate metabolism. Interestingly, a sugar kinase was also upregulated, presumably facilitating the phosphorylation of imported sugars for glycolytic entry, alongside an aldo/keto reductase, which may contribute to sugar alcohol interconversions or detoxification processes. In summary, the upregulation of these enzymes indicates that *R. bromii* enhances sugar transport and central metabolic processing when growing on soluble starch, which is more readily accessible than resistant starch, offering new insight into how *R. bromii* discriminates between soluble and resistant starch despite their shared chemical linkages.
We have now added these findings to the Discussion section (l. 520-527), and as Figure S18 and Table S6.

Introduction

Line 59 – this should be updated to say that only a few bacterial species have been identified as RS degraders within the human gut microbiota. There are others in other environments.
The text was modified accordingly.

There should likely be some note that for raw potato starch, B. adolescentis is generally found to be the most responsive. E.g. see https://doi.org/10.1016/j.nutres.2020.02.009
We have revised the text accordingly to reflect this specificity in bacterial adaptation to starch variations.

Line 129 probably should specifically note that it cannot use glucose
The text was modified accordingly.

Amylosome structure/composition

The Figure 1 legend notes that the amylosomes are linked to the cell wall through ‘elongated linkers’. What protein is serving as the linker between the bacterial cell and the amylosomes? Presumably this would be Sca2 and/or Sca5. Are these distances compatible with the size of these proteins?
We thank the reviewer for this question and for the opportunity to clarify our proposed amylosome model. Scaffoldins Sca2 and Sca5 serve as primary anchors of the amylosome to the cell wall. Both proteins bear a C-terminal sortase recognition motif, with an unstructured linker between the LPXTG sequence and the cohesin domain. We used AlphaFold3 (Abramson et al., 2024) modeling to predict domain sizes and linker lengths and found that

the Sca2 and Sca5 cohesin domains are located in distances compatible with those observed by cryo-ET.

To model the expected amylosome-cell wall spacing comprehensively, we further extended this analysis by predicting the structures of the additional 17 dockerin-containing proteins larger than 40 kDa, which were detected in the proteomics samples after growth on fructose or RS in the late stationary phase, i.e. under conditions matching those used for cryo-ET analysis. Considering an estimated length of 0.38 nm / amino acid, a fully extended linker of cohesin domains would comprise a distance of up to 35 nm (Sca2) or 64 nm (Sca5) from the cell wall. The globular densities we observe in our tomograms most likely represent the largest domains of the dockerin-containing proteins. We thus measured their predicted domain sizes and lengths of the unstructured linkers. These measurements revealed an average distance of the amylosome proteins 49 ± 15 nm from the cell wall with a half-extended linker, well within the observed particle distribution range.

We have included the above explanation in the revised manuscript (Fig. 1g and l. 184 - 192).

What mechanism could maintain a consistent ratio within the cell-free amylosomes, while allowing the composition of the cell-associated amylosomes to vary radically? This never really seems to be discussed.

We thank the reviewer for this important question. We can hypothesize that newly synthesized proteins are directly incorporated into the cell-associated amylosomes upon secretion, resulting in a dynamic and adaptive composition. In contrast, the cell-free amylosomes represent proteins that have been shed or released from the cell surface and may no longer be under active regulation; as such, their composition appears more stable and consistent. This hypothesis was now included in the discussion of the revised manuscript.

Line 238-241 is a bit worrying. If the components that are proposed to be essentially what defines a cell-associated amylosome are not being detected (even if they are in general confirmed to be present) the confidence in what is being detected being an unbiased representation would seem to be called into question.

We appreciate the reviewer's insightful comment. Indeed, the efficiency of our surface protein enrichment approach in releasing the covalently attached scaffoldins such as Sca2 and Sca5 may have been modest. However, this is likely related to inefficient proteolysis of the covalent protein to cell wall attachment. We thus expect the non-covalent interactions dominating the amylosome, such as protein-protein interactions, to be considerably less affected. In summary, we do not expect this to have significantly impacted the proteomic analyses in our study. We clarified this notion in the results section of the revised manuscript (l. 273 - 278).

The pullulan induced amylosome not including any of the pullulanases seems odd. This is clearly suboptimal for the bacteria, so what is driving this?

The reviewer is correct in pointing out this apparent discrepancy. As shown in Fig. S6 and Fig. S7, the amylosome pullulanase Amy12 is present in the cell-associated amylosome after growth on pullulan up to the late logarithmic phase. Both Amy10 and Amy12 were identified in the supernatant fractions of cultures grown on pullulan at all timepoints, indicating that these enzymes are indeed produced under these conditions (see Table S2).

This suggests that while pullulanases may not be consistently integrated into the cell-associated amylosome complex at all stages, they are expressed and secreted during growth on pullulan. *R. bromii* could import the maltooligosaccharides that Amy10 and Amy12 produce from pullulan in the supernatant, and thus not require their inclusion in the cell-associated amylosome. We are now referring to this notion in the Results section (l. 262 - 266) to avoid further confusion.

General

Line 198-201 I'm not sure I understand the link between these proteins and human health promotion.

We thank the reviewer for flagging this section as potentially confusing. We have removed it from the revised manuscript.

Line 269-271 No mention of amylopectin when discussing the structure of starch? Even high amylose varieties are 15% or (usually) more amylopectin.

We have now added a section discussing the differences between amylose and amylopectin to the results section, in the context of Fig. 3 (lines 303 - 309).

Figure 6 – I'm not really sure what value this figure is providing. Figure S15 seems to be a much more useful representation of the findings, maybe just focused on the resistant starch part. Possibly could be combined with 6b. 6a, seems better suited to being part of figure 1 as part of the explanation of the results seen there or as a supplemental that is discussed in conjunction with figure 1.

We thank the reviewer for the constructive criticism of the previous Fig. 6. We have followed the recommendations, and replaced it by a schematic model that summarizes our findings (Fig. 6, Fig. S17).

Enzyme structure and activity

Line 316-318 So the suggestion is that Amy4 is in fact a reducing end specific glucosidase? To make this claim would require additional biochemical characterization. Looking at activity towards a range of substrates (including those with a blocked reducing end) and ensuring that glucose is the main product released would be necessary.

We agree with the reviewer and we have removed this speculative claim from the revised manuscript.

I'm also not sure what the point of comparing Amy4 (amylase) and Amy10 (pullulanase) is here. There are many other amylase and pullulanase structures that would need to be included to conduct such an analysis properly. It also doesn't really seem to be relevant as it is already clear that they have different substrate preferences. A comparison between the Amy10 and Amy12 structures would seem to be more relevant to possibly identify differences that could lead to different substrate/product profiles of the two enzymes.

We thank the reviewer for pointing out the structural comparisons described in this study. We have now revised Figure 3 and the corresponding section to better delineate the scope of our analysis. This part of our manuscript provides a structural hypothesis for the observed functional differences between the CAZymes. Comparison of their active sites provides a molecular explanation for the activities of these proteins. Our initial structural comparison of Amy10 and Amy12 revealed that they were highly conserved in their structure, with a root-mean-square deviation of only 1.1 Å across 428 Cα atoms in the resolved domain. Nevertheless, a key difference between the two enzymes is the placement of the first MucBP domain, which is positioned closer to the active site for Amy10 than for Amy12 (Fig. 3c).

While we agree that the breadth of structures available from the GH13 family permits a much more thorough analysis of the regions determining substrate specificity, this has been done elsewhere for α(1,4)- and α(1,6)-specific enzymes (Møller et al., 2016; Okuyama et al., 2016). In both cases, the Trp residue which we highlight was identified as a key player in substrate coordination. We are thus confident we are showcasing a relevant structural difference between Amy4 and Amy10, and remain intrigued about the marked difference of Amy16 from their mold, both functionally and structurally.

Figure S8, which provides the information for Figure 3b and the interpretation of the activity of Amy16. I'm not really convinced of much of anything by those TLC figures. To demonstrate alpha1,6 activity you would specifically need to see maltotriose production, not just an indication of activity. The open active site of Amy16 is reminiscent of the B. thetaiotaomicron SusG enzyme, which has activity against pullulan, but functions as a neopullulanase, cleaving

the alpha1,4 bonds within pullulan. The open active site architecture allows it to accommodate branch points close to the alpha1,4 bond that it cleaves, but does not allow direct hydrolysis of alpha1,6 bonds. Additional characterization is needed to identify the products from pullulan hydrolysis (e.g. panose = neopullulanase, maltotriose = pullulanase). This can be done by TLC, with better standards and more care, but HPAEC-PAD would be the gold standard

We thank the reviewer for the excellent experimental suggestion which we have followed. To confirm our interpretation of the activity profile of Amy16, we performed HPAEC-PAD analysis of the degradation products of pullulan or maltoheptaose incubated with Amy16. We found that Amy16 accumulates small, but significant amounts of glucose, maltose, maltotriose and panose from pullulan, indicating it is active against α(1,6)-glycosidic bonds, albeit at a much lower level than against α(1,4) glycosidic bonds. We added additional panels in Figure 3c, Figure S9a, as well as their results in the main text (lines 332 - 344). This experiment highlights the unique dual role of Amy16.

Figure S10 and S11 both appear to show all of the details for Amy12 purification and structure determination, whereas the legend for S10 suggests that ii should contain the details for Amy10.

We apologise for this error in the supplementary figures. We have now inserted the figure showing the details of Amy10 purification and its structural determination as S11.

Substrates

How were the substrates prepared? Was the RS treated the same as for the growth experiments? Any heat treatment? For RS it is customary to subject them to mock upper intestinal digestion (eg. INFOGEST) to mimic what substrates the bacteria would actually encounter in the large intestine. Additionally to make any conclusions about activity towards RS, a number of different RS substrates should be tested (they vary quite dramatically in physical properties that are highly likely to influence enzymatic activity).

We thank the reviewer for pointing out the possible impact that pre-treatment has on the structure of RS, and the impact that RS structure has on the accessibility of bacterial enzymes to the polysaccharide chain. To test the impact of the autoclaving step required for bacterial culture on RS accessibility in our *in vitro* assay, we now performed side-by-side degradation assays of autoclaved and non-autoclaved HiMaize 958 by the five amylosome CAZymes. This revealed a substantially higher accessibility of the autoclaved substrate, with release of reducing sugars detected for Amy4, Amy9, Amy10 and Amy16, exceeding the amount released on non-autoclaved substrate 7 - 18-fold, depending on the enzyme. While our results show that non-autoclaved HiMaize is indeed a much more challenging substrate to degrade,

the synergistic interaction between Amy4 and Amy16 is still observed on the non-autoclaved substrate, further underscoring the importance of this enzyme synergy in RS degradation. The corresponding data were added to Figure S14a and the main text (l. 406 - 414).

Media and growth

how were the media prepared, were they all autoclaved? The Hi-Maize starches are relatively high gelatinizing, but from what I can find H-Maize 958 has a peak gelatinization temperature of just under 100 Celsius. So if subjected to a standard autoclave procedure it would be gelatinized. So more details are needed.

We thank the reviewer for this valuable point. All media, including those containing Hi-Maize starch, were autoclaved, as described in previous studies. We acknowledge that autoclaving Hi-Maize 958 alters its structural properties—specifically, it significantly reduces its native RS2 content and leads to the formation of a mixed RS2/RS3 material, or predominantly RS3 depending on the cooling conditions. We added this clarification to the revised Methods section.

The M2 media is undefined, containing for instance clarified rumen fluid and yeast extract. Could these components contain factors (e.g. carbohydrates) that are activating the expression of some of the amylosome components, masking differences between the carb sources used in this study?

We thank the reviewer for this important point. In all experiments for proteomics analysis, the bacteria were cultured on the same batch of M2 (base media) and only the carbon source was changed, ensuring that differential protein expression could be directly and exclusively attributed to the carbon source. Moreover when inoculated to M2 medium without supplementation of carbon source, the bacterium does not show growth, indicating that the undefined components, such as clarified rumen fluid and yeast extract, are not sufficient as sole carbon sources to sustain growth. These notions reduce the likelihood that any factor except for the carbon source influenced amylosome expression. We have clarified this in the revised Methods section.

Reviewer #2 (Remarks to the Author):

This study presents a comprehensive and methodologically robust investigation into the architecture and functional dynamics of the amylosome complex in Ruminococcus bromii, a key microbial species involved in resistant starch (RS) degradation in the human gut. The authors employ a multilayered approach—integrating cryo-electron tomography, proteomics,

and biochemical assays—to elucidate how structural and regulatory mechanisms govern enzymatic synergy and substrate degradation. The major finding of their work is its demonstration that amylosome function is modulated at two distinct levels: (1) spatial organization that enforces proximity among enzymes, and (2) condition-dependent expression shifts that alter enzyme composition. Notably, the study identifies Amy4 and Amy16 as dominant components under RS-rich conditions, accounting for 60% of the amylosome's enzymatic profile. Structural and functional analyses further reveal a synergistic interaction between these enzymes, enhancing RS breakdown and highlighting the organism's adaptive capacity to dietary fiber availability. Overall, this article is very well written and presented and makes a significant contribution to our understanding of microbial fiber degradation and its implications for colonic fermentation and host-microbiome interactions. The integrative methodology and mechanistic insights offer a valuable framework for future studies on gut microbial ecology and metabolic engineering. I have the following comments for consideration:
We thank the reviewer for appreciating the subject and the content of our study. We have addressed the reviewer's insightful comments throughout the manuscript, as indicated below.

Structure

In the section detailing the enzyme structure there are many predictions made as to the specificity of the CAZymes based on the size and shape of the catalytic binding site/cleft. When reading this the thought arises to actually test these substrates against Amy4 and Amy16, which of course the authors do in the following section. Is there not a way to slightly modify the text to at a minimum make this connection between the two bodies of work obvious to the reader when discussing the structures.
We have rephrased the results section and modified the layout of the figure covering our functional and structural analysis of the amylosome CAZymes to make our trajectory from domain annotation to validation of enzymatic activity to structural analysis more easy to follow.

It is also not clear if validating biochem assays were performed to confirm the predictions of linear vs branched substrates for say Amy10, 12 vs Amy4?
We thank the reviewer for raising this important point. We have indeed tested all five enzymes for activity against linear and branched substrates. Specifically, while Amy4 and Amy9 degraded only linear substrates, Amy10 and Amy12 degraded only branched substrates. In addition, we identified Amy16 as a promiscuous glycoside hydrolase, which preferentially hydrolyses linear α(1,4)-glycosidic bonds, but can also act as a debranching enzyme, hydrolysing α(1,6) glycosidic bonds. These functional characteristics are now highlighted in Fig. 3b & 3c.

Finally, given the structural differences of say Amy4 and 16, yet their tight and essential association I am curious if the authors have any hypothesis as to how they interact and coordinate hydrolysis of a RS substrate?

We agree that the precise synergistic interactions within the amylosome is a fascinating question. Although this will be addressed in a follow up project, we currently have two key hypotheses for this interaction:

- The increased overall number of CBMs in the interaction between Amy4 and Amy16 could lead to improved recruitment of the substrate. Similar avidity effects have been observed for CAZymes carrying many CBM domains.
- The combination of the α(1,4)-degrading activity of Amy4 and Amy16, and the additional α(1,6)-degrading activity of Amy16 could allow them to simultaneously act on the same polysaccharide chain, thereby increasing substrate accessibility.

We have included these ideas in the Discussion section, l. 556 - 561.

Regulation

When reading the manuscript I was curious if the authors had any insight into the key molecular triggers when a given substrate is exposed to the cell. In gram- PULS there is very often a two-component system (TCS), which are signal transduction pathways that allow cells to adapt to environmental changes. The triggers are often the corresponding disaccharide from the target substrate. When reading the final discussion/conclusions I see the author do in fact address this and state it is unknown. Nonetheless, I do wonder if there are any indicators in the gene organisation and/or the proteomes that indicate how the cells differentiate and respond to the changing substrates?

We appreciate the reviewer's thoughtful question regarding the molecular triggers that initiate the substrate-specific response in *R. bromii* and have addressed this point in the discussion section of the revised manuscript (lines 507 - 520). While Gram-negative polysaccharide utilization loci (PULs) often rely on two-component systems (TCSs) to sense environmental carbohydrates, Gram-positive organisms appear to employ distinct signaling strategies. In *Clostridium thermocellum*, for example, membrane-associated anti-sigma (anti-σ) factors regulate alternative sigma-I (σ^I) factors in response to extracellular polysaccharides (Bahari et al., 2011; Chen et al., 2023; Kahel-Raifer et al., 2010; Li et al., 2023; Muñoz-Gutiérrez et al., 2016; Nataf et al., 2010; Ortiz de Ora et al., 2018; Wei et al., 2019). In this context, we specifically searched for PF23750 domain-containing proteins in the *R. bromii* genome but

found no clear equivalents. This suggests that *R. bromii* may utilize a unique, yet-to-be-characterized regulatory system.

Text comments

On line 198 the authors state: “These included an annotated fructose-1,6-bisphosphatase, part of the gluconeogenesis pathway, and two annotated ATPases, potentially linking RS degradation to human health promotion through cross-feeding and the release of beneficial metabolites.” It is not clear to me how the identification of a common gluconeogenesis enzyme and ATPases links RS degradation to human health promotion. Either some more context needed here or some adjustment of the sentence.

We thank the reviewer for this comment. For clarity and to avoid any confusion, we have now removed this statement from the manuscript.

The authors use proteome abundances and % to great detail to explain the amylosome, a short clear concise sentence how they did this in the main text could be beneficial to the reader (it is detailed clearly in the methods)

We added a sentence to the Results section accordingly (l. 249 - 252).

Reviewer #3 (Remarks to the Author):

This manuscript presents an integrative study of the amylosome in Ruminococcus bromii, a multi-enzyme complex involved in resistant starch (RS) degradation. Combining cryo-electron tomography, cryo-EM, proteomics, and biochemical assays, the authors aim to elucidate the structural and functional basis of RS metabolism. While the study generates extensive datasets, the central mechanistic narrative, focusing on spatial synergy and functional complementarity, remains speculative and insufficiently supported by the current analyses. Better data integration, further experimental validation, and more restrained conclusions would substantially improve the manuscript.

We thank the reviewer for taking the time to thoroughly read our manuscript, and for providing us with the opportunity to improve and clarify the central narrative.

Major Comments:

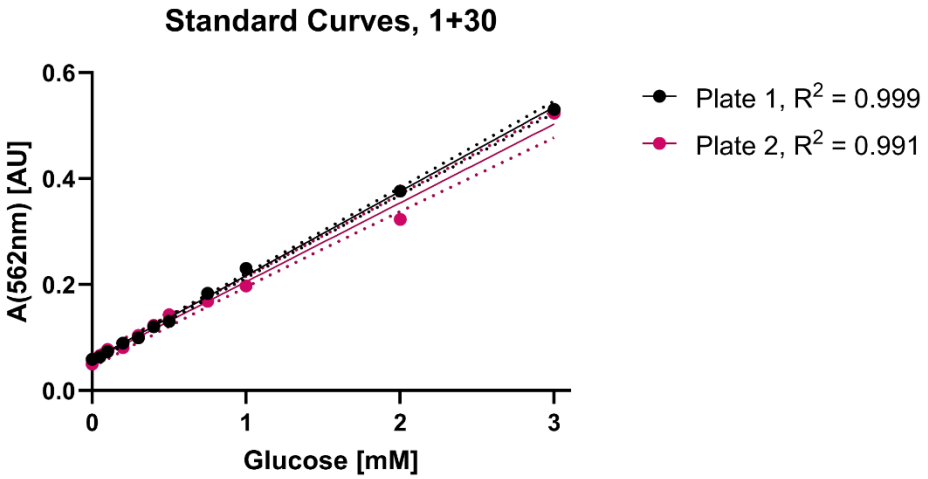
1. The central hypothesis remains descriptive:

The authors claim that “spatial constraints” within the amylosome drive enzyme synergy—is conceptually intriguing but remains speculative due to a lack of mechanistic evidence. While cryo-ET reveals extracellular densities and proteomics shows Amy4 and Amy16 enrichment on RS, these observations do not demonstrate direct spatial proximity. There is no experimental validation of physical interactions between enzymes on the native amylosome. SEC suggests that the complex forms, but further validation is necessary— e.g., freezing SEC fractions on EM grids to see if the structure of the complex can be determined, or using crosslinking-MS, proximity labeling. Otherwise, you may revise the narrative to reflect the current limitations in mechanistic insight.

We thank the reviewer for pointing this out to us. Using SEC followed by SDS-PAGE, we indeed show changes in retention of the individual proteins when they are co-injected into the column with subsequent co-elution of Amy4Δdoc and Amy16. These data indicate that these proteins interact *in vitro*, consistent with earlier data showing interaction of their cohesin and dockerin domains (Mukhopadhya et al., 2018). We have now added the SDS-PAGE gel to Fig. 5 showing that the co-elution peak contains Amy4 and Amy16 at similar proportions, in order to strengthen our claims of *in vitro* interaction. We thus state in the corresponding section that an *in situ* interaction of Amy4 and Amy16 could only be inferred from their abundances and binding affinity, but has not been directly shown in these studies (l. 458 - 470).

Synergy between Amy4 and Amy16 is shown via TLC and BCA assay, but no enzyme kinetics are provided. Also, have you confirmed that the substrate concentration in the BCA assay falls within the linear detection range? If not, this may lead to misinterpretation of the observed synergistic effect.

We thank the reviewer for suggesting these important controls. Regarding the linear range of the BCA assay. Indeed, we have verified that the substrate concentration is within a linear response range. Specifically, we carefully calibrated the dilution of the reaction mixture to measure within the linear range of the assay. We included appropriate standards in each individual set of BCA assay to fit a per-plate calibration curve, with typical R^2 values > 0.99. As an example, we include below the standard curve that underlies the measurements shown in Fig. 4b-d.



With regard to enzyme kinetics, as HiMaize RS is insoluble, we could not perform classical Michaelis-Menten analysis of reaction kinetics, which requires knowledge of the accessible substrate concentration in solution. However, we sampled the product formation over time to validate that we were capturing a state of substrate abundance, and to measure whether the observed synergistic interaction varies over time. This experiment was added to Fig. S14b and is described in the results section in l. 431 - 434.

2. Cryo-ET lacks in-depth analysis:

The tomographic data are interesting but not fully mined. Only 21 tomograms are analyzed in detail. The blob-picking strategy is vaguely described and does not clearly define what is being measured. The STA approach is only shown for a ribosome control but fails to extract meaningful information from the amylosome layer, likely due to heterogeneity. This point is not critically discussed. Include quantitative segmentation or mapping of amylosome density across the cell perimeter to support claims of architectural regularity. Consider focused classification attempts (e.g., using EMAN2 or Warp/M) on amylosome-associated densities—even unsuccessful attempts would help clarify limitations.

We thank the reviewer for the opportunity to elaborate on our cryo-ET data analysis. We acquired a large dataset encompassing hundreds of tomograms, but used in this manuscript

only the highest quality of data for our analysis of amylosome architecture. The large dataset was used for conducting STA using the software packages that were mentioned by the reviewer. However, the small size of the amylosome proteins (average 68 kDa, range 10-150 kDa) and their structural similarities, resulted in modest resolution that does not allow a meaningful interpretation.
We have added more details about our attempts at resolving and classifying the densities at these coordinates by STA, both in the Results and Discussion section, and as Fig. S3.

3. Proteomics dataset is extensive but underinterpreted:

The proteomic dataset is extensive but lacks rigorous interpretation. The criteria for "RS-specific" proteins are inconsistent: sometimes defined by presence/absence (Jaccard), other times by intensity ranks. Several major observations (e.g., Amy16 increase) are reported descriptively but not statistically validated at the protein level (only at the core proteome level). The PCA and ANOVA are presented, but not linked to biological conclusions. Apply differential expression analysis (e.g., LIMMA or DEqMS) to identify significantly upregulated proteins across RS vs. other substrates. Clarify how rankings and fold changes were calculated. A volcano plot or heatmap with adjusted p-values would add interpretive rigor.

We thank the reviewer for this valuable comment which helped us strengthen our conclusions. According to the reviewer's suggestion, we have added analyses and reorganized the section to clearly distinguish between different layers of analysis: (i) general observations on the global proteomes across carbon sources (including both supernatant and cell-associated fractions), (ii) targeted in-depth analysis of the cell-associated amylosomes, which comprise the focus of our study, and (iii) focused evaluation of CAZyme composition within amylosomes.

- ANOVA was applied to highlight global trends in proteome structure, highlighting that the expressed proteome size varied significantly with the carbon source (90% of the variance explained, $p < 0.001$) but was largely unaffected by the growth phase (2% of variance explained, $p = 0.0019$).
- Jaccard analysis was used to evaluate presence/absence across conditions, allowing us to account for the total number and identity of proteins detected across carbon sources without relying on intensity data. This approach was necessary because the proteomic dataset derives from two separate fractions (supernatant and cell-associated), prepared using distinct sample processing methods, which limits the validity of direct quantitative comparisons. In both fractions, the protein expression

clustered significantly by carbon source (cell-associated: ANOSIM R = 0.71, p = 0.0001, supernatant: ANOSIM R = 0.66, p = 0.0001).

- PCA was used to show that the cell-associated amylosome composition varied significantly with the carbon source (ANOSIM R = 0.65, p = 0.0001).
- We have added LIMMA-analysis to highlight proteins that are significantly upregulated in resistant starch compared to other carbon sources (Figure S6, Table S3). This analysis identified Amy16 as significantly upregulated on RS, reinforcing our conclusions regarding the importance of this enzyme in RS degradation, now also discussed in the text (l. 244 - 245).

We have now revised the text to better integrate these statistical analyses with the biological interpretations.

Minor comments:

•Several figures (e.g., Fig. 2, Fig. S5) are dense and lack adequate legends or in-text guidance. Subpanels are not cited consistently, and it's hard to follow which datasets support which claims. Simplify main figures, provide better in-text figure references (e.g., “Fig. 2c, right panel”), and summarize key quantitative findings in tables.

We thank the reviewer for the advice on improving the readability of the manuscript. We have added subpanel titles where possible (e.g. in Fig. 2), and worked on improving the in-text references.

•While loop differences and pocket accessibility offer insight, they do not fully account for dynamic substrate binding. The conclusion about substrate promiscuity versus specificity might be premature without substrate-bound structures or molecular dynamics simulations.

We agree with the reviewer that a single-factor explanation of enzyme activity based on an apo structure can not fully account for all aspects of biological complexity. We have thus rephrased our claims more carefully.

•Have you mutated the C-terminal loop (e.g., H383/W384 in Amy4) to test its role in substrate specificity?

We thank the reviewer for this interesting and appealing idea. Nevertheless, due to concerns about potentially disrupting the overall fold of the protein, we think that this would be existing avenue for further future research.

•A more detailed comparison of the active sites of Amy4 and Amy16 is needed to clarify how structural differences contribute to their distinct substrate preferences. This includes analyzing specific loop architectures and residue-level interactions that may affect active site accessibility.

We have changed Figure 3 to include a comparison of Amy4, Amy10 and Amy16. In particular, we have highlighted the residues that might play a key role in active site accessibility.

Reviewer #4 (Remarks to the Author):

I co-reviewed the paper with Dr. Xiao-Ru Chen and she submitted our comments today 5/28/25. I thank you for the opportunity. The paper is good and interesting but it needs some work and polishing from the authors.

We thank both co-reviewers for the constructive evaluation of our manuscript.

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We thank the reviewers for providing their thoughtful suggestions throughout the review process. We feel that the experiments suggested during the initial review substantially improved the manuscript, and we are glad to see that the reviewers agree with these changes.

Please find below a point-by-point response to the reviewer's comments:

Reviewer #1:

The authors have addressed all of my concerns. I will only leave the additional small comment, that while the transcriptional profile is different between the soluble starch and resistant starch, the fact that none of the amylosome components show up in this analysis, suggest that the amylosome compositional differences are not controlled by transcriptional regulation. However, elucidating the full mechanism by which they are controlled, is likely beyond the scope of this manuscript.

We thank the reviewer for this additional comment. We changed the Discussion section to emphasize this thought (l. 532 - 536).

Reviewer #3:

The new data and clarifications have substantially improved the manuscript. The addition of statistical and structural analyses, biochemical results, and a clear acknowledgment of the limitations of cryo-ET and in situ data strengthens the work. The synergistic effect of Amy4 and Amy16 is convincingly demonstrated.

I have only minor suggestions:

- Proteomics data show strong enrichment of Amy9 on RS, yet biochemical assays indicate little activity against RS. While the manuscript clarifies differences between autoclaved and non-autoclaved starch, the precise role of Amy9 in the amylosome remains unclear. Based on the predicted Amy9 structure or existing literature, the authors could briefly speculate on its potential function and why it does not show a synergistic effect, or simply acknowledge this as an open question for future research.

We thank the reviewer for pointing out this interesting open question. We have added a sentence acknowledging that the differential activity profiles of Amy9 and Amy16 despite their sequence similarity remain an open question (l. 387-390)

- You note that the cell-wall anchored scaffoldins Sca2 and Sca5 were not consistently detected in the proteomics data. This is an important detail. You could make the

connection between the transcriptomics and proteomics results more clear, perhaps by highlighting this as a limitation of the current study to provide a complete picture of amylosome regulation.

We thank the reviewer for this suggestion and modified the discussion section to reflect this limitation as also requested by Reviewer 1 (l. 532 – 536).

- There is a typo in Figure 2 legend: “Crbon” → “Carbon.”

We have fixed the typo.