

1 **Gut Microbial Utilization of the Alternative Sweetener, D-**  
2 **Allulose, via AlsE**

3

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## 15 **Abstract**

16 D-allulose, a rare sugar with emerging potential as a low-calorie sweetener, has garnered  
17 attention as an alternative to other commercially available alternative sweeteners, such  
18 as sugar alcohols, which often cause severe gastrointestinal discomfort. D-allulose-6-  
19 phosphate 3-epimerase (AlsE) is a prokaryotic enzyme that converts D-allulose-6-  
20 phosphate into D-fructose-6-phosphate, enabling its use as a carbon source. However,  
21 the taxonomic breadth of AlsE across gut bacteria remains poorly understood, hindering  
22 insights into the utilization of D-allulose by microbial communities. In this study, we  
23 provide experimental evidence showing that *Clostridium innocuum* is capable of D-  
24 allulose metabolism via a homologous AlsE. A bioinformatics search of 85,202 bacterial  
25 genomes identified 116 bacterial species with AlsE homologs, suggesting a limited  
26 distribution of AlsE in bacteria. Additionally, *Escherichia coli* contains a copy of *alsE*, but  
27 it does not grow on D-allulose as a sole carbon source unless *alsE* is heterologously  
28 expressed. A metagenomic analysis revealed that 15.8% of 3,079 adult healthy human  
29 metagenomic samples that we analyzed contained *alsE*, suggesting a limited prevalence  
30 of the enzyme in the gut microbiome. These results suggest that the gut microbiome has  
31 limited capacity to metabolize D-allulose via *alsE*, supporting its use as an alternative  
32 sweetener with minimal impact on microbial composition and gastrointestinal symptoms.  
33 This finding also enables personalized nutrition, allowing diabetic individuals to assess  
34 their gut microbiota for *alsE*, and manage glycemic response while reducing  
35 gastrointestinal distress.

## 36 **Introduction**

37           The obesity epidemic is a serious health issue affecting many countries  
38 worldwide.<sup>1</sup> According to the National Health and Nutrition Examination Survey  
39 (NHANES), conducted by the National Center for Health Statistics (NCHS), 41.9% of U.S.  
40 adults aged 20 and older are obese.<sup>2</sup> As an individual's amount of adipose tissue  
41 increases, so too does their risk for metabolic diseases, including type 2 diabetes,<sup>3</sup> which  
42 is caused by insulin resistance and lack of insulin, resulting in chronic hyperglycemia.<sup>4</sup>  
43 Over 415 million people worldwide suffer from diabetes, over 90% of whom have type 2  
44 diabetes.<sup>5</sup> In the U.S., 14.8% of adults aged 20 or older are also affected.<sup>2</sup>

45           Previous studies have linked increased sugar consumption to the obesity and  
46 diabetes epidemic.<sup>6,7</sup> Further, researchers propose that a high-carbohydrate diet  
47 promotes the deposition of calories into fatty tissue, leading to weight gain through  
48 increased hunger.<sup>8</sup> The main culprits of type 2 diabetes are excessive sugar consumption  
49 and a sedentary lifestyle.<sup>5</sup> There is no cure for type 2 diabetes available as of 2024, and  
50 much more research is needed on methods to mitigate and prevent diabetes, including  
51 decreasing the consumption of fructose, glucose, and sucrose.

52           One potential strategy to minimize sugar consumption is to use sugar substitutes,  
53 such as aspartame, sucralose, erythritol, xylitol, and sorbitol.<sup>9</sup> These alternative  
54 sweeteners tend to taste sweet, but the human body does not metabolize them, thereby  
55 reducing the adverse health effects of excess sugar consumption.<sup>10</sup> These sweeteners  
56 may be derived from plant extracts or from chemical synthesis.<sup>11</sup>

57           Many of the alternative sweeteners currently approved by the U.S. Food and Drug  
58 Administration include sugar alcohols and non-nutritive sweeteners (NNS), both of which

59 have been associated with some side effects on the human gut microbiome. Sugar  
60 alcohol consumption can lead to gastrointestinal discomfort and have laxative effects  
61 through osmotic pressure and increased gas production through gut bacterial  
62 fermentation, resulting in diarrhea and bloating.<sup>12</sup> Moreover, increased blood erythritol  
63 levels have been associated with increased platelet reactivity, resulting in cardiovascular  
64 events such as strokes.<sup>13</sup> On the other hand, regular NNS consumption can lead to  
65 functional alteration of gut microbiota composition, resulting in an impaired glycemic  
66 response and glucose intolerance.<sup>14,15</sup> Therefore, it is extremely important to understand  
67 the mechanisms of how alternative sweeteners interact with the human gut microbiome.  
68 These findings have led to an increasing interest in fructose epimers, sugar molecules  
69 that resemble fructose but have altered stereochemistry at one carbon atom.<sup>16</sup> One  
70 example is D-allulose (also known as D-psicose), a rare low-calorie sweetener that is the  
71 C-3 epimer of fructose and is found in small amounts in certain fruits.<sup>16</sup> Previous studies  
72 have suggested that D-allulose has a low glycemic index, making it promising for reducing  
73 the risk of diabetes.<sup>17-20</sup> Due to advances in the industrial process and bacterial  
74 engineering methods, D-allulose production is becoming increasingly economically  
75 viable.<sup>21</sup> Thus, D-allulose is a promising way to decrease sucrose and fructose  
76 consumption.

77         Although D-allulose is a promising alternative sweetener, its side effects are poorly  
78 understood compared to other types of alternative sweeteners. A significant portion of  
79 ingested D-allulose reaches the gut microbiome, as approximately 30% passes through  
80 the small intestine unabsorbed and is excreted in feces<sup>22,23</sup>. While 70% of D-allulose is  
81 absorbed via glucose transporter type 5 (GLUT5) in the small intestine, the substantial

82 unabsorbed fraction has the potential to interact with and impact the gut microbial  
83 community.<sup>22,23</sup> Studies in murine models have shown that D-allulose can induce changes  
84 in the gut microbiome.<sup>24,25</sup> Comparatively, Suez et al. 2014 showed that saccharin,  
85 sucralose, and aspartame can induce glucose intolerance through modifications of the  
86 gut microbiome composition and function.<sup>26</sup> Therefore, there is an urgent need to better  
87 understand both the potential for D-allulose utilization by gut bacteria and its effects on  
88 human gut microbiome composition.

89         Some bacteria possess the ability to metabolize D-allulose using the enzyme D-  
90 allulose-6-phosphate 3-epimerase (AlsE), encoded by the gene *alsE*.<sup>27</sup> In *E. coli* K-12,  
91 *alsE* is in the D-allose operon, which has been well characterized (**Figure 1A**).<sup>28</sup> First, D-  
92 allose is converted into D-allulose 6-phosphate via AlsK and RpiB. Then, AlsE catalyzes  
93 the reversible conversion of D-allulose 6-phosphate to D-fructose 6-phosphate.<sup>29</sup>  
94 Environmental and clinical isolates of *Klebsiella pneumoniae*, an opportunistic pathogen  
95 responsible for a significant number of nosocomial bacterial infections,<sup>30</sup> are capable of  
96 metabolizing D-allulose using a homologous AlsE, raising concerns that consuming D-  
97 allulose may confer opportunistic pathogens an advantage in colonization.<sup>31,32</sup> However,  
98 there has not been a full systematic annotation of the prevalence and distribution of *alsE*  
99 in the human gut microbiome.

100         To address this gap, we combined bioinformatic predictions and experimental  
101 verification to characterize the distribution of *alsE* in human gut microbes. Our  
102 bioinformatic predictions were validated through growth experiments, culturing bacteria  
103 in media with D-allulose as the sole carbon source. Our investigation of multiple  
104 representatives of the major gut bacterial clades expanded the known phylogenetic range

105 of D-allulose metabolism from phylum Pseudomonadota to include phylum Bacillota by  
106 identifying that *Clostridium innocuum* 6\_1\_30 is capable of using D-allulose as a sole  
107 carbon source. Through comparative genomics and protein homology searches, we  
108 identified a putative AlsE in *C. innocuum* that is homologous to AlsE in *K. pneumoniae*  
109 (35% identity, e-value 2.89e-41) that has a divergent operon organization compared to  
110 the known D-allulose metabolizers. We verified the function of the *C. innocuum* AlsE in  
111 the *E. coli* Keio Knockout Collection, observing that *E. coli* deficient in native *alsE* was  
112 able to grow on D-allulose when complemented with *C. innocuum alsE*. We also found  
113 that *E. coli*, despite encoding *alsE*, cannot grow on D-allulose as a sole carbon source  
114 unless *alsE* is heterologously expressed. To comprehensively characterize the taxonomic  
115 distribution of AlsE, we performed a systematic search across 85,202 bacterial genomes,  
116 identifying 116 species encoding putative *alsE* homologs. The limited distribution of *alsE*  
117 in the gut microbiome supports D-allulose's promise as an alternative sweetener with  
118 minimal impact on both microbial composition and gastrointestinal symptoms, two  
119 common drawbacks of current artificial sweeteners. Although our focus is on *alsE*, it is  
120 important to note that there could be alternative undiscovered pathways bacteria can use  
121 to metabolize D-allulose that our study did not cover. These findings provide insights into  
122 bacterial D-allulose metabolism, supporting its development as an alternative sweetener  
123 to help reduce sugar consumption in the context of rising rates of obesity and diabetes.

## 124 **Results**

### 125 **Investigating the potential for D-allulose utilization by gut microbes**

126 To identify gut bacterial species capable of utilizing D-allulose as a carbon source,  
127 we conducted a preliminary identification of species with D-allulose-6-phosphate 3-

128 epimerase (AlsE) homologs by conducting a BLASTp search against 85,202 non-  
129 redundant genomes from the Genome Taxonomy Database<sup>33,34</sup>. We used the  
130 experimentally verified *Klebsiella pneumoniae* AlsE as the query, with a threshold of 50%  
131 identity and bitscore greater than 200. There were 272 species that met the threshold,  
132 mostly from non-gut bacteria. Some gut bacteria genera with AlsE homologs include  
133 *Klebsiella*, *Escherichia*, and *Clostridium*. Interestingly, *Clostridium innocuum*, a common  
134 gut bacterium species, contained an AlsE hit (50.49% identity, 2.30e-77 e-value, 220  
135 bitscore).

136 To experimentally validate our bioinformatic predictions and characterize D-  
137 allulose metabolism across diverse gut bacteria, we tested representative strains from  
138 major gut bacterial phyla including Bacillota, Bacteroidota, Actinobacteriota, and  
139 Pseudomonadota for growth on D-allulose as a sole carbon source (**Figure 2A**). Growth  
140 was quantified by spectrophotometric measurement at OD600, with significant growth  
141 defined as a three-fold increase in OD600 compared to media negative controls.  
142 *Clostridium innocuum* 6\_1\_30 demonstrated robust growth on D-allulose with a 6:1 ratio  
143 in its OD600 measurement compared to the media blank, revealing a previously unknown  
144 metabolic capability (**Figure 2B**). Notably, *Escherichia coli* DC10B did not grow on D-  
145 allulose despite encoding *alsE* within its D-allose operon (**Figure 2C**), prompting further  
146 investigation.

#### 147 ***Escherichia coli* does not readily utilize D-allulose *in vitro* despite encoding *alsE***

148 Although *E. coli* encodes *alsE* within the D-allose operon (*alsRBACEK*), previous  
149 studies have demonstrated that this operon is specifically induced in response to D-

150 alllose.<sup>28</sup> We hypothesized that while *E. coli* encodes the metabolic machinery for D-  
151 allulose utilization; this capability may not be active in the absence of D-allose.

152 To test this hypothesis, we placed *alsE* under the control of an IPTG-inducible  
153 promoter to enable controlled expression independent of its native regulation. Using the  
154 Keio collection, a comprehensive library of single-gene knockout mutants in *E. coli*  
155 BW25113<sup>35</sup>, we cloned the *alsE* gene from strain JW2760 into a pCW-lic vector backbone  
156 under an inducible *tac* promoter, creating the pCW-lic-*E. coli alsE* construct. This plasmid  
157 was transformed into the Keio *alsE* knockout strain, and the transformed bacteria were  
158 cultured in M9 minimal media supplemented with D-allulose and IPTG.<sup>12</sup>

159 Our results showed that both the Keio *alsE* knockout and the untransformed *E. coli*  
160 were unable to grow using D-allulose as the sole carbon source. In contrast, the  
161 transformed *E. coli* overexpressing *alsE* exhibited robust growth (**Figure 3A and 3B**).  
162 These findings support our hypothesis that while *E. coli* encodes AlsE, it may not be  
163 capable of utilizing D-allulose as a carbon source in the absence of D-allose.

#### 164 **Limited distribution of *alsE* across *E. coli* strains**

165 We then investigated the presence and absence of *alsE* across *E. coli* genomes,  
166 using a previously published pangenome consisting of 1,324 *E. coli* genomes.<sup>36</sup> *alsE* was  
167 present in 598 out of 1324 *E. coli* genomes (45%), suggesting that *alsE* is a strain-specific  
168 gene and may not be present in every individual's gut microbiome, despite the prevalence  
169 of *E. coli* exceeding 90% among in humans.<sup>37</sup>



## 170 **Identification of *alsE* in *Clostridium innocuum***

171         Given that *Clostridium innocuum* 6\_1\_30 grew on D-allulose as a sole carbon  
172 source, we investigated the genomic origins of its D-allulose metabolism. Based on our  
173 prior preliminary search results, we hypothesized that a homologous *alsE* was  
174 responsible for D-allulose metabolism in *C. innocuum* rather than a novel pathway. To  
175 conduct a comprehensive search for AlsE homologs in *C. innocuum*, we used the  
176 *Klebsiella pneumoniae* MGH 78578 AlsE (NCBI accession: GCA\_000016305.1) as the  
177 query to search the *C. innocuum* genome (NCBI accession: GCA\_000183585.2).  
178 BLASTp revealed two AlsE homologs in *C. innocuum*, referred to as ci04257 and ci04568  
179 (ci04257: 50.49% identity, 2.09e-76 e-value; ci04568: 35% identity, 2.89e-41 e-value).  
180 We examined the gene neighborhood of the two *alsE* candidates. The neighborhood of  
181 ci04257 consisted mainly of genes encoding hypothetical proteins. On the other hand,  
182 ci04568 was adjacent to phosphotransferase systems (PTS), which could potentially  
183 perform the phosphorylation and import step of D-allulose utilization. In addition, the  
184 neighboring genes are annotated with sugar metabolism functions, such as fructose  
185 bisphosphate aldolase. Therefore, we hypothesized that ci04568 encodes an enzyme  
186 that possibly performs a similar function to AlsE. Interestingly, the putative *alsE* gene  
187 neighborhood in *C. innocuum* is completely divergent from the *alsE* gene neighborhood  
188 in other species known to metabolize D-allulose, such as *Klebsiella pneumoniae* (**Figure**  
189 **1B**).<sup>31</sup> Of note, this putative *alsE* was a core gene present in all 283/283 available *C.*  
190 *innocuum* genomes on NCBI, with all of them containing a nearly identical, if not identical,  
191 homolog to *alsE* in 6\_1\_30.

192 We then sought to functionally validate the candidate *alsE* in *C. innocuum* by  
193 cloning the gene into a pCW-lic vector backbone under an inducible tac promoter,  
194 resulting in the pCW-lic\_C.inn\_alsE construct to heterologously express *C. innocuum*'s  
195 *alsE* in *E. coli* (**Supplemental Figure 1**). The plasmid was then transformed into the Keio  
196 collection *E. coli alsE* knockout. The transformed bacteria were subsequently inoculated  
197 in D-allulose-supplemented M9 and induced *alsE*'s expression using IPTG.<sup>12</sup> The Keio  
198 *alsE* knockout demonstrated no growth on D-allulose, while complementation of the *C.*  
199 *innocuum* gene into the knockout restored function, resulting in growth on D-allulose  
200 (**Figure 3C and 3D**).

#### 201 **Few Gut Bacterial Species Encode AlsE**

202 Once we experimentally verified the function of the *C. innocuum* and *E. coli* AlsE,  
203 we used ProkFunFind,<sup>38</sup> a bioinformatics pipeline to systematically search for AlsE in  
204 bacteria. We used the experimentally verified AlsE protein sequences from *K.*  
205 *pneumoniae* MGH 78578, *C. innocuum* 6\_1\_30, and *E. coli* K-12 as queries to search the  
206 85,202 non-redundant prokaryotic genomes from the Genome Taxonomy Database  
207 (GTDB) for species that contained homologs to AlsE. We used a more stringent filtering  
208 criteria compared to the preliminary search, filtering hits based on a 30% identity  
209 threshold and a maximum e-value of 1e-100. Our search revealed 116 putative bacterial  
210 species with AlsE (**Supplemental Table 3, Figure 4**). The vast majority of these species  
211 were from the phylum Pseudomonadota (103/116), 10 were from the phylum Bacillota,  
212 and 3 were from Fusobacteriota. Out of those 116 species, only 35 are known to be part  
213 of the animal gut microbiota. Some known members of the human gut microbiome with  
214 AlsE include *Klebsiella oxytoca*, *Enterobacter cloacae*, and *Serratia marcescens*. Other

215 species with AlsE that are not gut-associated are primarily isolated from plants and soil,  
216 including *Klebsiella planticola*,<sup>39</sup> *Rahnella aquatilis*,<sup>40</sup> and members of the *Kosakonia*  
217 genus.<sup>41,42</sup> Of note, none of the species that were unable to grow on D-allulose during our  
218 initial investigation contained AlsE homologs, with the exception of *Escherichia coli* as  
219 discussed before (*Bacteroides cellulosilytious*, *Lactobacillus reuteri*, *Clostridium*  
220 *symbiosum*, *Bifidobacterium adolescentis*, *Ruminococcus gnavus*).

### 221 **Presence of *alsE* in the healthy adult gut microbiome**

222 To investigate the prevalence of *alsE* in the human gut microbiome, we examined  
223 the presence and absence of *alsE* in 3,079 healthy adult human gut microbiomes that  
224 passed quality checks. We built a reference database using both experimentally  
225 characterized and bioinformatically discovered *alsE*, with thresholds of 30% identity and  
226 1e-100 e-value. We then aligned healthy adult human stool metagenomic reads  
227 downloaded from SRA to our *alsE* reference database and normalized the alignment  
228 counts into counts per million. To strike a balance between spurious hits and sensitivity,  
229 we considered any metagenomes with at least 1 count per million to contain *alsE*. 488  
230 out of 3,079 metagenomes met our threshold for *alsE* presence, approximately 15.8%.

### 231 **Delineation of AlsE from Pentose-5-Phosphate 3-Epimerase**

232 In order to elucidate the evolutionary origins of AlsE, we used a combination of  
233 phylogenetic analyses, ancestral state reconstruction, and sequence conservation. Using  
234 eggNOG-mapper (v6.0), we determined that AlsE belonged to the orthologous group  
235 COG0036 (pentose-5-phosphate 3-epimerase). We constructed a phylogenetic tree  
236 using the top 1,323 homologs of the 3 experimentally verified AlsE protein sequences  
237 against all COG0036 sequences. Based on branch lengths and the presence of the

238 experimentally verified AlsE, we identified a putative AlsE clade that contains 515  
239 sequences. We were able to identify conserved amino acid changes in the putative AlsE  
240 node from the ancestral node (**Figure 5A**), such as from G52 to S52, V134 to Y134, L142  
241 to T142, and an N147 to D147 (**Figure 5B**). Based on the high entropy in the alignment  
242 at these positions, these are conserved changes and may differentiate AlsE from other  
243 pentose-5-phosphate 3-epimerases.

244 To determine putative catalytic residues, we performed a structural alignment of  
245 the AlphaFold2-predicted structure of *C. innocuum* AlsE to the crystal structure of *E. coli*  
246 AlsE (pdb: 3CT7). Based on the active site residues reported by Chan et al. 2008 in *E.*  
247 *coli* AlsE, we determined that the putative active site residues in both *Clostridium*  
248 *innocuum* and *Klebsiella pneumoniae* are similar based on the structural alignment  
249 (**Figure 5C**).<sup>29</sup> In *C. innocuum*, these putative residues are His 32, Asp 35, His 66, and  
250 Asp 175, which align to His 34, Asp 36, His 67, and Asp 176 in *E. coli*, respectively.  
251 Therefore, despite being distant homologs, *C. innocuum* likely shares similar catalytic  
252 residues to *E. coli* AlsE.

## 253 Discussion

254 Many widely used commercial alternative sweeteners, such as sugar alcohols, are  
255 associated with significant gastrointestinal discomfort.<sup>12</sup> This discomfort arises from the  
256 malabsorption of these sweeteners, leading to osmotic diarrhea, and from fermentation  
257 by gut microbes, which produce gas.<sup>12</sup> Consequently, there is an urgent need to identify  
258 alternative sweeteners, such as D-allulose, that do not cause gastrointestinal symptoms.

259 The presence of gut bacteria that can potentially metabolize D-allulose via D-  
260 allulose-6-phosphate 3-epimerase (AlsE) has significant implications for its use as a  
261 commercial alternative sweetener. Prior to our study, while D-allulose metabolism had  
262 been identified in some human gut bacteria, there had not been a systematic analysis of  
263 the presence, abundance, and distribution of enzymes involved in D-allulose metabolism  
264 across gut bacterial species - a knowledge gap that limited our understanding of how gut  
265 bacteria utilize this sweetener. In our study, we demonstrated that *Clostridium innocuum*  
266 can metabolize D-allulose through a homologous AlsE by examining its growth on D-  
267 allulose media. These findings shed light on the role of the gut microbiome in D-allulose  
268 metabolism.

269 During the initial investigation for gut microbial species capable of growing on D-  
270 allulose as a sole carbon source, *C. innocuum* 6\_1\_30 grew on D-allulose as a sole  
271 carbon source while *E. coli* was unable to grow despite encoding *alsE* in its genome,  
272 which was intriguing. Past studies have shown that despite *E. coli* having *alsE* in its  
273 genome, its expression was too weak to support the production of D-allulose from D-  
274 fructose without genetic modifications.<sup>21,43</sup> This is consistent with our findings that the *E.*  
275 *coli* only grew on D-allulose when *alsE* was heterologously expressed, verified via the

276 insertion of the respective *alsE* genes into the Keio *alsE* knockout mutant, which resulted  
277 in *E. coli* gaining the ability to use D-allulose as a sole carbon source.

278 Our findings show that AlsE protein homologs are only present in a few gut  
279 bacterial species. Out of 85,202 bacterial genomes from the GTDB, only 116 bacterial  
280 species were annotated to contain AlsE homologs. Our finding that *E. coli* cannot grow  
281 on D-allulose without heterologously expressing *alsE* suggests that some of these 116  
282 species may not be able to metabolize D-allulose effectively. In addition, only 35 of these  
283 species are known to be present in animal gut microbiomes. These data suggest that D-  
284 allulose utilization might be restricted to a small number of species within the human gut  
285 microbiome. This finding is in alignment with the scarcity of D-allulose in nature. D-  
286 allulose has only been found in small quantities in a few plant species, such as *Itea*  
287 *virginica* and wheat.<sup>44,45</sup> Moreover, several of the bacterial species with putative *alsE* were  
288 primarily isolated from plants such as wheat or maize, including *Klebsiella planticola*,<sup>39</sup>  
289 *Rahnella aquatilis*,<sup>40</sup> and members of the *Kosakonia* genus.<sup>41,42</sup> We speculate that D-  
290 allulose metabolism may confer a metabolic advantage for these bacteria that live in  
291 plant-associated habitats, where exposure to D-allulose is more likely. Alternatively, *alsE*  
292 may have evolved primarily to confer D-allose metabolism, with D-allulose metabolism  
293 being incidental.

294 Notably, the limited presence of *alsE* in gut microbiome species suggests that D-  
295 allulose may serve as a valuable alternative to common sugar substitutes, which are  
296 known to cause gastrointestinal discomfort and alter microbiome composition. Previous  
297 studies have reported that D-allulose can be consumed in relatively high doses, up to 0.5  
298 g/kg body weight, without causing significant gastrointestinal issues.<sup>46</sup> Thus, the limited

299 metabolism of D-allulose by gut bacteria, combined with its low impact on gastrointestinal  
300 function, suggests that it may offer a promising solution for individuals seeking low-calorie  
301 sweeteners without adverse digestive effects. Of note, approximately 15.8% of human  
302 metagenomes analyzed contained *alsE*, suggesting that individual gut microbiomes may  
303 respond differently to D-allulose consumption. Diabetic individuals looking to cut their  
304 glucose consumption may benefit from individual microbiome testing to choose the  
305 alternative sweetener that is less likely to be utilized by their gut microbiome.

306 Our study focuses on AlsE as an enzyme responsible for D-allulose metabolism,  
307 though we recognize the possibility of alternative mechanisms of D-allulose metabolism.  
308 To our knowledge, AlsE is the only currently known enzyme implicated in D-allulose  
309 metabolism in bacteria. However, there may be alternative mechanisms of bacterial D-  
310 allulose metabolism that are undiscovered, given the limited studies on the subject.<sup>28,31</sup>  
311 Due to this possibility of unknown alternative mechanisms, we cannot be certain of D-  
312 allulose's impact on gut microbiome composition at large.

313 In conclusion, we shed light on the taxonomic distribution of AlsE in the gut  
314 microbiota. We discovered that *C. innocuum* is capable of growing on D-allulose as a sole  
315 carbon source. In addition, while *E. coli* has *alsE*, it cannot grow on D-allulose without  
316 heterologously expressing *alsE*, suggesting that many of these bacteria do not  
317 necessarily grow on D-allulose as a sole carbon source. A relatively small fraction of gut  
318 microbes are capable of utilizing D-allulose, making it a promising alternative to  
319 commercially available sugar substitutes, such as sugar alcohols.

## 320 **Methods**

321 *Identification of D-allulose-6-phosphate 3-epimerases in the GTDB genomes:* All  
322 representative genomes from the Genome Taxonomy Database (GTDB) (release r207)  
323 were downloaded, and protein sequences for each genome were predicted using Prokka  
324 (version 1.14.6).<sup>34,47</sup> The *Escherichia coli* K-12, *Klebsiella pneumoniae* MGH78578, and  
325 *Clostridium innocuum* 6\_1\_30 D-allulose-6-phosphate 3-epimerase protein sequences  
326 were searched against 85,202 reference genomes using the ProkFunFind pipeline  
327 (v0.1.0).<sup>38</sup> The hits were filtered based on an 1e-100 e-value and 30% identity thresholds,  
328 resulting in putative 126 AlsE amino acid sequences from 116 nonredundant genomes.

329

330 *Phylogenetic analyses:* Sequences from the GTDB assigned to COG0036 were identified  
331 using eggNOG-mapper (version 2.1.3).<sup>48</sup> A BLASTp search was conducted (version  
332 2.15.0+) using the identified D-allulose 6-phosphate 3-epimerases as queries against  
333 these identified sequences, setting a limit to the top 1,305 hits. Sequence alignment was  
334 performed using Clustal Omega (version 1.2.4).<sup>49,50</sup> Columns that have more than 97%  
335 gaps were removed to enhance alignment quality using Galign (version 0.3.7).<sup>51</sup>  
336 Phylogenetic analysis was carried out using IQ-TREE (version 2.1.2) with default  
337 parameters and model selection<sup>52</sup>. The reliability of the phylogenetic trees was evaluated  
338 using 1,000 ultrafast bootstrap replicates. Trees were visualized using the Interactive  
339 Tree Of Life (iTOL).<sup>53</sup>

340 Ancestral sequence reconstruction was performed on the AlsE tree using GRASP  
341 (version 04-May-2023), with default parameters.<sup>54</sup> We then manually inspected the tree  
342 to delineate AlsE from other Pentose-5-phosphate 3-epimerases. We calculated the



343 entropy of the alignments using Goalign via the compute pssm function (v.0.3.7).<sup>51</sup> The  
344 figures were created using the Python package logomaker (v0.08).<sup>55</sup>

345

346 *Growth of anaerobic bacteria:* Bacterial strains were acquired from the NIH Biodefense  
347 and Emerging Infections Research Resources Repository (BEI). Each strain was  
348 inoculated from a glycerol stock and grown under anaerobic conditions over a 24-hour  
349 period at 37 °C in an anaerobic chamber (Coy Laboratory Products) in Brain-Heart  
350 Infusion (BHI) broth (Research Products International, B11000) supplemented with  
351 glucose. 25 µL of the culture was inoculated into 4 mL of minimal media (M9)  
352 supplemented with 10 mg/mL carbon source (glucose or D-allulose).<sup>12</sup>

353

354 *Absorbance Assay:* The transformed Keio  $\Delta alsE::C. Innocuum alsE$  & Keio  $\Delta alsE::E. coli$   
355 *alsE* constructs were shaken in Luria-Bertani (LB) supplemented with 100 µg/mL  
356 carbenicillin (GoldBio, C-103-25) overnight at 37°C. 25 µL of the overnight culture was  
357 inoculated in 4 mL triplicates of minimal media (M9) supplemented with 100µM Isopropyl  
358 β- d-1-thiogalactopyranoside (IPTG, GoldBio, I2481C25), 100 µg/mL carbenicillin, 50  
359 µg/mL kanamycin (Bio Basic, KB0286), and 10 mg/mL D-allulose<sup>12</sup> (Chem-Impex,  
360 32353). For kinetic measurements, 250 µL of the triplicates were aliquoted into a 96-well  
361 acrylic, clear bottom plate (Celltreat, 229592), sealed with a Breathe Easy membrane  
362 (Electron Microscopy Sciences, 70536-10), and incubated at 37°C for 48-70 hours  
363 depending on the strain observed. The end-point absorbance at 600 nm was measured  
364 with a Spectramax M5 plate reader, with end-point bacterial growth calculated using a  
365 ratio to the blank, with a ratio of 3 indicating significant growth.

366

367 *pCW-lic\_C.inn\_alsE* & *pCW-lic-E.coli\_alsE* constructs: In order to achieve ectopic  
368 expression of *alsE* from *Clostridium innocuum* 6\_1\_30 and *E. coli* JW2760<sup>35</sup> in the  
369 knockout mutants, the *alsE* gene was amplified and cloned into the pCW-lic vector  
370 backbone (Addgene, 26098). Genomic DNA from *C. innocuum* and *E. coli* JW2760 was  
371 utilized in a polymerase chain reaction (PCR) using Phusion High-Fidelity DNA  
372 Polymerase (NEB, M0530S) with the specific primers listed in Supplementary Table 1. A  
373 Monarch PCR & DNA Cleanup Kit (NEB, T1030S) was used to purify the amplified  
374 product. The pCW-lic vector backbone was digested with restriction enzymes NdeI (NEB,  
375 R0111S) and HindIII-HF (NEB, R3104S), followed by purification with a Monarch PCR &  
376 DNA Cleanup Kit. A Gibson assembly was completed using Gibson Assembly Master Mix  
377 (NEB, E2611S) in accordance with the manufacturer's instructions. The resulting  
378 constructs were stored at -20°C until needed for use.

379

380 *Keio-pCW construct*: The *alsE* gene was amplified and cloned into the pCW-lic vector  
381 backbone under a tac promoter and transformed into the Keio collection *alsE* knockout  
382 as detailed above with the same primers outlined in Supplementary Table 1. For the  
383 control, an empty pCW-lic vector was cloned into the Keio *alsE* knockout.

384

385 *Chemical competency*: The Keio collection *alsE* knockout was made competent using the  
386 Mix & Go! *E. coli* Transformation Kit and Buffer Set (Zymo, T3001) in accordance with the  
387 manufacturer's protocol and stored at -80°C until needed for use.

388

389 *Transformation:* Both constructs were independently transformed into the chemically  
390 competent Keio collection *alsE* knockout in accordance with the manufacturer's protocol  
391 (Zymo, T3001). The resulting transformed cells were plated on LB agar plates  
392 supplemented with 100 µg/uL of carbenicillin. Successful transformation was validated  
393 via Oxford Nanopore sequencing by Plasmidsaurus.

394

395 *Structural prediction and molecular docking:* The structure for the *Clostridium innocuum*  
396 6\_1\_30 AlsE was predicted using AlphaFold2 (v2.3.0).<sup>56</sup> Binding pockets were predicted  
397 using fpocket (v4.0) with default parameters.<sup>57</sup> The pockets were compared to the  
398 homologous *Escherichia coli* AlsE (3CT7) to identify putative substrate binding regions  
399 and catalytic residues.<sup>29</sup> The structure for D-allulose (PubChem compound identifier:  
400 50909805) was docked onto the predicted AlsE structure using AutoDock Vina (v4.2).<sup>58,59</sup>  
401 The docking simulation was performed within 15 Å × 15 Å × 15 Å cubes centered on the  
402 center points of the chosen fpocket substrate binding pocket with exhaustiveness set to  
403 32. Docking results were visualized using PyMOL.<sup>60</sup> We used Foldseek to identify the top  
404 structural homolog.<sup>61</sup> The predicted AlsE protein structure was aligned with the *E. coli*  
405 3CT7, and the putative catalytic residues were identified based on the previous work by  
406 Chan et al. 2008, using TM-Align.<sup>29,62</sup> Protein sequence conservation of AlsE was  
407 visualized using ConSurf based on the putative AlsE clade.<sup>63,64</sup>

408

409 *Profiling of alsE presence in the gut:* To build the reference database, we used *alsE*  
410 identified by ProkFunFind, which were filtered based on a threshold of e-value 1e-100  
411 and percent identity 30%, resulting in a total of 126 sequences. We downloaded a

412 collection of adult healthy metagenomic biosamples that passed basic quality control  
413 (n=3410) from SRA, and then trimmed adapters with Trim-Galore with default settings.  
414 The reads were then mapped to a human reference (assembly T2T-CHM13v2.0) to  
415 identify potential contaminants and removed them using Samtools (v1.16).<sup>65</sup> We removed  
416 any samples with less than a million reads after curation, resulting in 3,079 samples, and  
417 then aligned the remaining reads to the *alsE* reference database using bowtie2 (v2.4.1).<sup>66</sup>  
418 The number of reads mapped to the *alsE* reference was summarized by normalizing the  
419 number of reads in the sample and then multiplying by one million to obtain counts per  
420 million (cpm). If a biosample had multiple SRRs, we concatenated the read counts and  
421 total reads across all SRRs per sample before calculating cpm. We considered samples  
422 with at least 1 cpm as containing *alsE*, to account for spurious alignments.

423

## 424 **Author Statements**

425 **Author contributions:** B.H. and X.J. conceptualized and supervised the project. All  
426 authors performed the experiments and analyzed the data. G.M.N, A.J., C.R., and M.G.  
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435

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437 <http://n2t.net/addgene:26098> ; RRID:Addgene\_26098)

438 **Data and materials availability:** The authors confirm that the data supporting the

439 findings of this study are available within the article and its supplementary materials.

440

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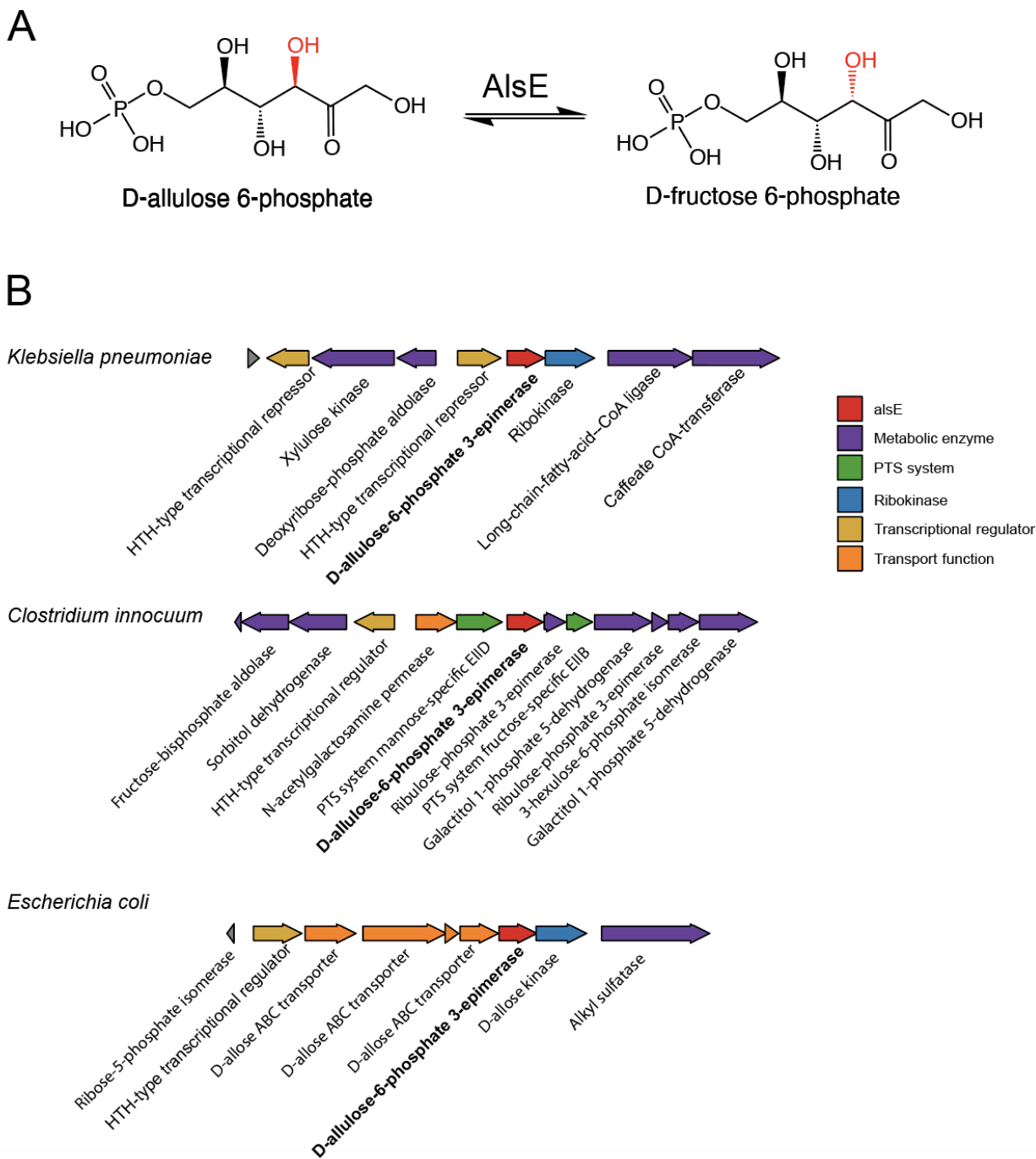


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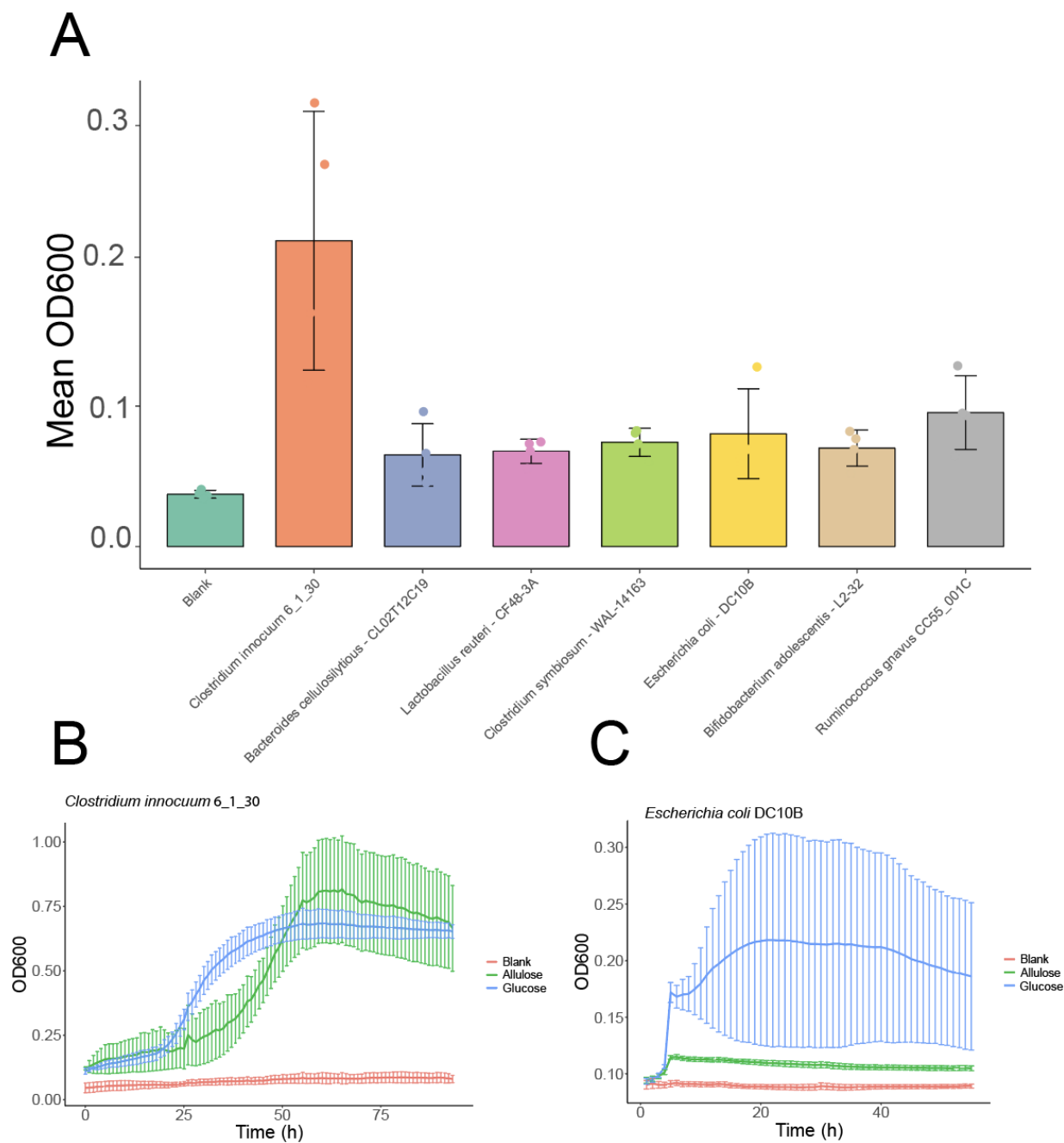
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596 **Figures**



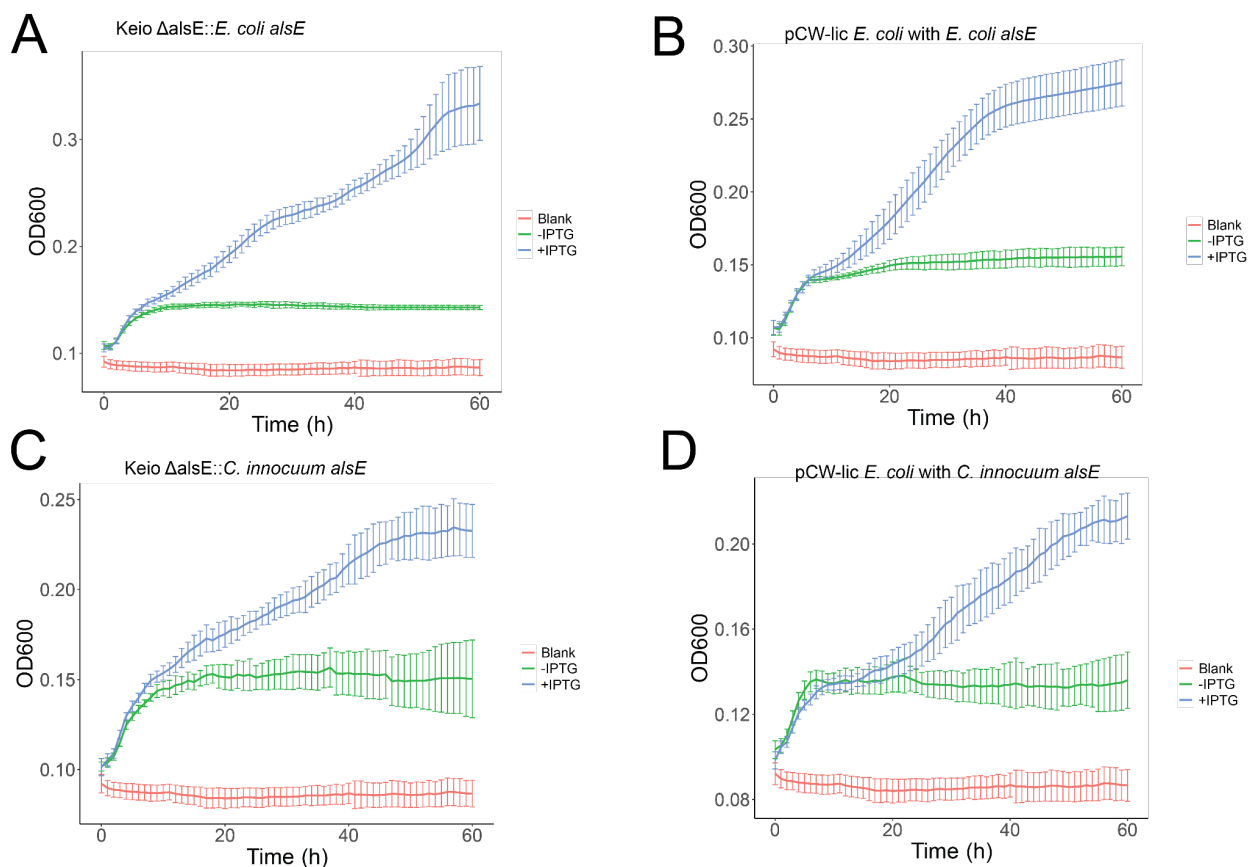
597  
 598 **Figure 1:** A) Conversion of D-allulose-6-phosphate into D-fructose-6-phosphate by *alsE*. B) *alsE*  
 599 cluster organization from *Klebsiella pneumoniae* MGH78578, *Clostridium innocuum* 6\_1\_30, and  
 600 *Escherichia coli* K-12.



601  
602 **Figure 2:** Identification of *Clostridium innocuum* 6\_1\_30 as a gut bacteria species that  
603 can grow on allulose as a sole carbon source. A) Investigation of 7 gut bacteria species  
604 (*Clostridium innocuum* 6\_1\_30, *Bacteroides cellulosilytious* - CL02T12C19, *Lactobacillus*  
605 *reuteri* CF48-3A, *Clostridium symbiosum* WAL-14163, *Escherichia coli* DC10B,

606 *Bifidobacterium adolescentis* L2-32, *Ruminococcus gnavus* CC55\_001C) for growth on  
607 allulose as the sole carbon source. Each data point is the average of three technical  
608 replicates from a single biological replicate per species. B) Growth curve of *C. innocuum*  
609 6\_1\_30 on allulose with minimal media. Allulose is the growth curve of *C. innocuum* when  
610 grown on allulose, while Glucose is a positive control of *C. innocuum* growing on glucose,  
611 and Blank refers to *C. innocuum* grown on blank media as a negative control. C) Growth  
612 curve of *E. coli* DC10B (Col02) on allulose with minimal media.

613



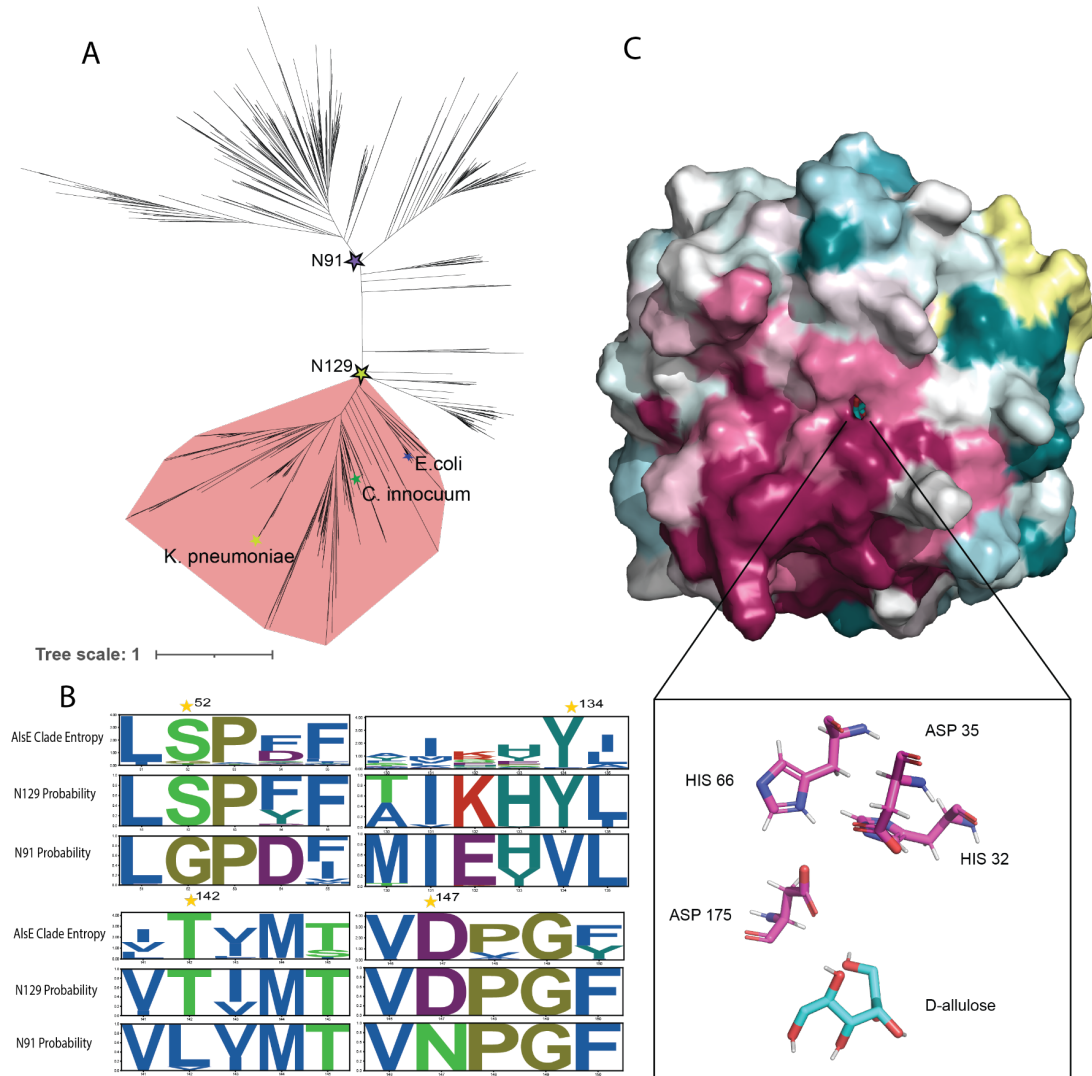
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615





626 **Figure 4:** Species tree showing the taxonomic distribution of AlsE in microbial genomes  
627 from GTDB, colored by order. The species tree was generated by pruning the GTDB  
628 species tree using the Gotree prune command.<sup>51</sup>  
629



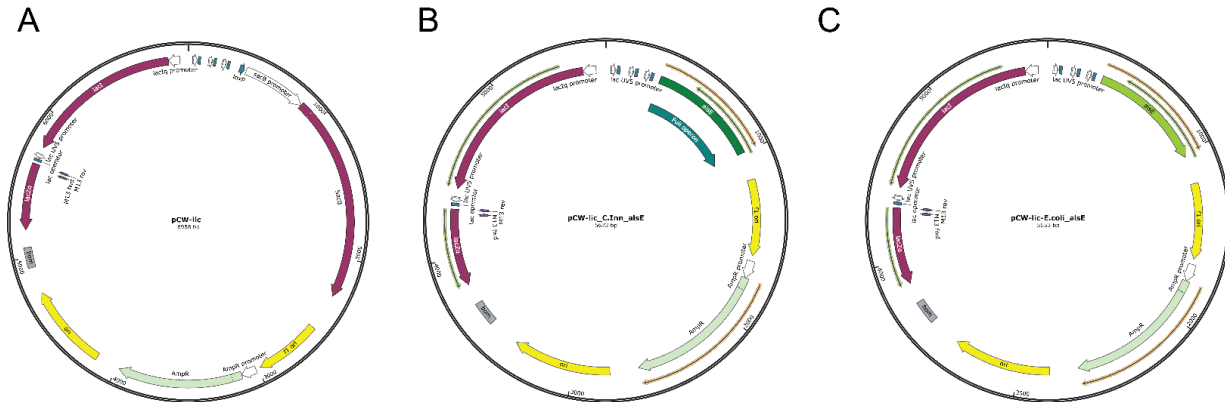
630  
631 **Figure 5:** A) Gene tree constructed from putative AlsE sequences and related enzymes  
632 annotated as pentose-5-phosphate-3-epimerase (COG0036), showing a possible  
633 delineation of the AlsE clade and the location of *Klebsiella pneumoniae*, *Clostridium*  
634 *innocuum*, and *Escherichia coli* AlsE. B) Diagram showing the change and conservation



635 (entropy) of residues in the putative AlsE clade, as well as the GRASP predicted ancestral  
636 states of N129 (AlsE clade) and N91 (putative ancestral node). Residues with a predicted  
637 conserved change from the ancestral state are labeled with a star. C) D-allulose docked  
638 to the AlphaFold2-predicted structure of *C. innocuum* 6\_1\_30 AlsE, colored by amino acid  
639 conservation via ConSurf.

640 **Supplementary Materials**

641



643 **Supplementary Figure S1:** Plasmid maps of constructs. A) pCW-lic vector backbone.31

644 B) pCW-lic\_C.inn\_alsE vector containing the D-Allulose-6-phosphate 3-Epimerase (*alsE*)

645 gene from *C. innocuum* ligated into the pCW-lic vector backbone via Gibson assembly.

646 C) pCW-lic-E.Coli\_alsE vector containing the *alsE* gene from *E. coli* ligated into the pCW-

647 lic backbone. Pale green arrows represent an ampicillin resistance gene. Maps created

648 using SnapGene.

649

650

651

652

653

654 **Supplementary Table 1: Primers used in construct development**

Strain		Sequence (5' > 3')
<i>C. innocuum</i> 6_1_30	Forward	CATCGATGCTTAGGAGGTCAAATGGAT ATAAAAATATCACCATCTATC
	Reverse	TTGACAGCTTATCAGCGATATTATTTTTC CAATTCCTGAAC
<i>E. coli</i> JW2760	Forward	CATCGATGCTTAGGAGGTCAAATGAAAA TCTCCCCCTCGTTAATG
	Reverse	TTGACAGCTTATCAGCGATATTATGCTG TTTTTGCATGAGGC

655

656 **Supplementary Table 2:** Predicted AlsE amino acid sequences identified with taxonomy  
657 information.

658 **Supplementary Table 3:** Metadata on the metagenomics samples used in this study,  
659 including SRA numbers and sample ids, along with the read mapping counts and total  
660 reads.