Context-dependent differences in the functional responses of Lactobacillaeca strains to fermentable sugars

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Abstract

Lactobacillaceae are Gram-positive rods, facultative anaerobes, and belong to the lactic acid bacteria (LAB) that frequently serve as probiotics. We systematically compared five LAB strains for the effects of different carbohydrates on their free-living and biofilm lifestyles. We found that fermentable sugars triggered a heterogeneous response in LAB strains, frequently manifested specifically in altered carrying capacity during planktonic growth and colony development. The fermentation capacities of the strains were compatible and could not account for heterogeneity in their differential carrying capacity in liquid and on a solid medium. Among tested LAB strains, *L. paracasei, and L. rhamanosus GG* survived self-imposed acid stress while *L. acidophilus* was extremely sensitive to its own glucose utilization acidic products. The addition of a buffering system during growth on a solid medium significantly improved the survival of most tested probiotic strains during fermentation. We suggest that the optimal performance of the beneficial microbiota members belonging to lactobacilli is heterogeneous and varies as a function of the growth model and the dependency on a buffering system.

Introduction

Firmicutes are dominant phylum in the human microbiota and the proportions of these phylum members vary between individuals and are greatly influenced by the local pH(1). Accordingly, the stomach is the least diverse growth niche within the human gastrointestinal tract (due to its extreme acidity). In the microbiome, the presence of probiotic strains varies between healthy individuals. Probiotics are defined simply as "microorganisms that when administered in adequate amounts confer a health benefit to the host" (2). Probiotic strains are consumed either as fresh fermentation products or as dried bacterial supplements with Lactobacillaceae and Bifidobacteria, being the two widely used probiotic species(3, 4). The main strains currently used for probiotic formulation were originally isolated from fermented products or humans(5–7).

Lactobacillaceae are Gram-positive rods, facultative anaerobes, and belong to the lactic acid bacteria (LAB) group, as lactic acid is their main end-product of carbohydrate metabolism(8, 9). This family is naturally found in the gastrointestinal tract (GIT) of humans and animals as well as in the urogenital tract of females(10). LAB are considered efficient fermenters, proficient in the production of energy under anaerobic conditions, or when oxygen is limited. The fermentation process involves the oxidation of carbohydrates to generate a range of products including organic acids, alcohol, and carbon dioxide(11). In general, the response of all LAB strains to fermentation is considered to be uniform and primarily depends the capacity to utilize glucose and its products (2).

In LAB, a phosphotransferase system (PTS) transports glucose across the membrane that is then used by the glycolysis pathway to produce pyruvate. This pathway generates energy and consumes NAD^+. Pyruvate is then converted into L- and D-lactate by the stereospecific NAD-dependent lactate dehydrogenases (LDHs), LdhL and LdhD, respectively, which regenerate NAD^+ and maintain the redox balance(12) . In the presence of oxygen and following the depletion of glucose, lactate is oxidized to pyruvate via the Lactate Oxidase enzyme followed by the production of acetate using pyruvate oxidase and ACK enzymes. The formation of acetate as the major fermentation end product, results in homoacetic fermentation (13). In addition to the importance of glucose utilization to microbial metabolism, glucose and fructose induce biofilm formation in *lactobacillus rhamnusos GG,* the most characterized LGG strain due to changes in the protein abundance and major changes in the surface proteome,

63 indicating that changes in carbon sources and their concentrations induce more
64 complex adaptations than alterations in microbial growth (14). Whether these
65 carbohydrate utilization induces regulatory effects in additional LAB strains remain to
66 be determined.

67 To map adaptations to carbohydrates that are growth dependent and independent, we
68 systematically compared the response to metabolic stress in microaerophilic (CO_2
69 enriched environments) >3% CO^2) conditions of the five probiotic Lactobacillaceae
70 species: *Lacticaseibacillus rhamnosus GG* (15)*, Lacticaseibacillus casei* (16)*,*
71 *Lactobacillus acidophilus* (17)*, Lacticaseibacillus paracasei,* and *Lactiplantibacillus*
72 *plantarum* (18) during planktonic growth and colony biofilm formation. *Bacillus*
73 *coagulans*, a probiotic *Bacilli* belonging to the same phylum(19), was studied as a non-
74 Lactobacillaceae LAB control strain. Under our conditions, similar glucose utilization
75 efficiencies were observed between the species (as judged by the levels of the end
76 products: lactate and acetate). Our results indicate that a differential response to
77 carbohydrates is correlated with a differential acid tolerance, rather than differential
78 production of organic acids. Thereof, LAB bacteria significantly differ in their response
79 to their own self-imposed acid stress from glucose utilization products and can be
80 clustered into fermentation resistant and fermentation sensitive strains. The differential
81 adaptation to acidic products included reversible changes in cellular organization and
82 colony formation.

83

84 **Results**

85 **Fermentable sugars specifically but heterogeneously affect the carrying**
86 **capacity during planktonic growth**

87 To test whether fermentable sugars affect growth differently than non-fermentable
88 sugars, we grew five probiotic Lactobacillaceae species on a rich medium (TSB) in a
89 shaking culture either with glucose (fermentable) or with raffinose (non-fermentable).
90 All bacteria grew similarly in rich medium. The addition of glucose induced growth as
91 judged by an increased carrying capacity of *L. acidophilus* and *LGG* compared to TSB
92 alone, but its effect on growth *in L. paracasei*, *L. casei L. plantarum and B.coagulans*
93 was extremely noisy (Figure 1). To better distinguish between growth patterns of the
94 different probiotic strains, we further analyzed the growth with the software

95 GrowthRates 3.0. We extracted the length of the lag phase, the growth rate and the
96 carrying capacity of all LAB strains with the different treatments (20). Our results
97 indicated that the heterogeneous response is primarily manifested in an altered
98 carrying capacity (e.g.: the maximal OD of the cultures) (Figure 2A). Strains showed
99 varied carrying capacity from each other, and indeed *L. acidophilus* and *LGG* had
100 enhanced carrying capacity in response to Glucose, while other probiotic strains did
101 not exhibit significant response. In all species the growth rate and lag time were not
102 significantly altered by glucose addition (Figures 2B and C).

103 We then asked whether the induction of growth is an outcome of fermentable sugar
104 utilization, as fermentation is a metabolic process allowing the production of energy
105 under anaerobic conditions. To answer that question, we assessed the growth of the
106 bacteria on a medium supplemented with raffinose, a sugar source less compatible
107 with fermentation, as it was indicated that alpha-galactosidase, responsible for the
108 hydrolysis of this sugar, is not enzymatically active (21–23). Raffinose did not induce
109 the growth of LGG, suggesting that the induction of growth depends on the
110 fermentable nature of the sugar (Figures 1 and 2A). In contrast, the carrying capacity
111 of *L. acidophilus* cultures increased with both glucose and raffinose (Figure 1) but
112 failed to meet statistical analysis of each parameter of growth (Figures 2B and C). In
113 general, the addition of raffinose failed to induce significant alterations in growth
114 parameters in all strains. Similar results were observed when the bacteria were grown
115 with mannose (fermentable) that induced an enhanced carrying capacity and xylose
116 non-fermentable), failing to induce significant alterations of growth (Figure S1). With
117 the exception of carrying capacity, other parameters of growth remained unaltered by
118 sugar application In all strains (Figures 1, 2 and S1).

119

120 **Differential response of Lactobacillaceae to fermentation is not a readout of**
121 **organic acid production**

122 To compare the fermentation efficiency, we measured the acidity of the medium before
123 and after growth in the presence and absence of glucose. Indeed, after 24h the pH of
124 the medium decreased in both glucose and raffinose (Figure 3A). The acidity of
125 Lactobacillaceae conditioned medium grown in the absence of glucose was
126 approximately 5, while the addition of glucose to the medium lowered the pH to 4 or

127 less confirming that upon application of glucose, the acidification of the growth media
128 was enhanced in all Lactobacillaceae that were tested. Compared with other tested
129 strains, *B. coagulans* altered the pH more modestly, e.g.: to 4.5 in the presence of
130 glucose and 6 in its absence (Figure 3A). The pH drop of the growth medium source
131 was comparable between all Lactobacillaceae stains in glucose and raffinose, except
132 in LGG which was inert to raffinose. This result indicates that differential utilization of
133 glucose may not account for the differential carrying capacities of the cultures in the
134 presence of glucose.

135 In order to confirm that the uniform drop in pH levels results from the differential
136 accumulation of organic acids among the tested LAB species, we measured the
137 accumulation of glucose utilization products (organic acids), focusing on the levels of
138 key organic acids, lactate and acetate using High-performance liquid chromatography
139 (HPLC) (calibrated as in Figure S2). In general, lactic acid was ten times more
140 abundant than acetic acid in the growth media. When we statistically analyzed the
141 concentration of lactate produced by the bacteria, there was no single species that
142 was significantly different from the other group members, indicating that the production
143 of organic acids and thereby glucose utilization is similarly performed under our
144 conditions (Figure 3B and C) and cannot account for the differential carrying capacity.

145 To exclude that the differential response to fermentation is a result of different
146 enzymatic activities of enzymes involved in the process, we studied *in silico* the
147 presence of lactate dehydrogenase from different LAB strains. Alignment of LDH
148 proteins from different Lactobacillaceae (Figure S3) indicated that *LGG L. paracasei*
149 and *L. casei* have two groups of homologues LDH proteins with more than 90%
150 identity. 16S alignment showed that all species have at least 89% similarity, and the
151 evolutionary closest species are *LGG L. casei* and *L. paracasei* based on 16S.
152 Therefore, the key differences revealed by growth analysis on fermentable sugars are
153 despite the high evolutionary closeness of all five species.

154

155 **Self-imposed acid stress in biofilm colonies of LAB species**

156 To further study the differential response to fermentation in Lactobacillaceae, we
157 examined the growth on solid medium under static conditions, which is frequently
158 correlated with biofilm formation(24). To test whether the addition of fermentable

159 sugars plays a role when grown on solid media under microaerophilic conditions, we
160 assessed the colony morphology with the addition of glucose and raffinose (Figure
161 4A). In all strains, the application of glucose-induced the formation of asymmetric,
162 smaller, and morphologically different colonies. Raffinose did not induce the same
163 morphological changes in the colony structure, suggesting glucose metabolism has a
164 role also in shaping the colony architecture (Figure 4A). Solid growth media from
165 bacteria grown on TSB or TSB with raffinose had similar pH values of approximately
166 5.5 (Figure 4B). The addition of glucose to the growth media lowered the pH
167 significantly suggesting that glucose-induced fermentation in all tested strains under
168 these conditions. To test whether acid stress is related to the alterations in colony
169 morphology we monitored cell death in the presence and absence of a buffering
170 system. As shown, in all strains, with the exceptions of *L. paracasei*, the buffering
171 system significantly enhanced the survival of the cells, and acid-dependent cell death
172 during fermentation significantly varied between the strains (Figure 4C-4D). The
173 addition of a buffer also resulted in colony morphology comparable to the morphology
174 of strains grown without glucose. While cell death was reduced, a buffer did not fully
175 restore the viability of the cells within a colony, it was sufficient to restore colony
176 morphologies to those observed in a glucose-free solid medium (Figure 4A). These
177 results may suggest that cell death on solid biofilm media primarily but not solely
178 results from differential acid sensitivity, and that adaptation to acidic stress triggers
179 alterations in biofilm formation capacities independently of cell counts. These results,
180 observed in all LAB strains are consistent with recent findings that the stress protein
181 Hsp plays an important role in shaping colony morphology under acidic pH in a single
182 specie: *L. plantarum* (25).

183 To better assess the level of stress on the single-cell level we grew the bacteria on
184 solid growth media (TSB), TSB with glucose, TSB with raffinose in the presence and
185 absence of a buffering system. Colony cells grown without glucose are rod-shaped,
186 divide normally, and are arranged in short chains (Figure 5). While all the species
187 looked similar in TSB, glucose-induced noticeable alterations in the morphology of
188 *LGG* and *L. paracasei* cells. Noticeable clumps were induced in both species, which
189 also lost their characteristic elongated rod shape. Alteration in cell shape upon glucose
190 treatment could be partially rescued with the application of buffer, supporting a
191 differential response of probiotic bacteria to fermentation and its acidic products.

192 Interestingly, alterations in cell shape did not perfectly correlate with the response to
193 acid stress as judged by cell growth as LGG had pronounceable growth upon
194 utilization of glucose, while *L. paracasei* failed to increase it carrying capacity with
195 glucose. Therefore, the alterations in cell shape may be independent adaption to
196 carbohydrate (for example a change in surface proteome) and not a direct reflection
197 of acid stress.

198

199 **Discussion**

200 Probiotic bacteria are considered a means for microbiota modulation, along with
201 nutrition, personal hygiene, and lifestyle (1). Probiotics are found to have a beneficial
202 effect on the consumer in the prevention of antibiotic-associated diarrhea and acute
203 infectious diarrhea, treatment of inflammatory bowel disease (26), and other gastric
204 disorders(27). Lactobacillaceae are widely used probiotic bacteria, represented in
205 most fermented products and supplements. Probiotic performance in the gut is
206 dependent on nutrient composition and availability (28). Positive effects on probiotics
207 proliferation and beneficial effects on the host have been associated with prebiotic
208 consumption. For example, it was shown that the addition of raffinose and *L.*
209 *acidophilus* to the diet of rats decreases their body weight and increases the
210 concentration of *L. acidophilus* in the gut (29). However, the responses of single LAB
211 strains to their own acidic fermentation products may vary and contribute to their
212 physiological responses to carbohydrates.

213 Therefore, we systematically compared five different LAB species for their response
214 to utilization of glucose and raffinose model sugars. We found that glucose utilization
215 has a general role in shaping the colony morphology under static conditions, as all
216 species exhibit morphological colony changes upon glucose treatment. Similarly,
217 medium acidification was generally similar during static growth (Figure 4B) and
218 comparable for all Lactobacillaceae during planktonic growth (Figure 2). For all tested
219 species, the final product of glucose utilization and fermentation (occurring under
220 microaerophilic conditions) was almost exclusively the organic acid lactate alongside
221 acetate. Analyses of fermentation capacities of the different species revealed similar
222 fermentation efficiencies. Lactic and acetic acid concentrations and the pH of the
223 growth medium were all similar and stable among the different species (Figures 3 and

224 4B). In agreement, the alignment of lactate dehydrogenase proteins revealed that
225 *LGG, L. paracasei* and *L. casei* have two groups of almost identical protein homologs
226 with more than ninety percent identity between the different species (Figure S3).

227 In contrary to glucose utilization, organic acid accumulation, and medium acidification
228 that are comparable between strains, the carrying capacity of the culture is
229 differentially enhanced during planktonic growth of Lactobacillaceae. While *LGG, L.*
230 *acidophilus L. casei* and *L. plantarum* doubled the carrying capacity following exposure
231 to glucose exposure, *L. paracasei* had no noticeable growth induction with glucose.
232 Interestingly, the capacity of LAB strains to utilize glucose, raffinose, mannose, and
233 xylose for planktonic growth differed significantly (Figures 1, 2, and S1). A detailed
234 analysis of the microbial growth reflected that carbon utilization is unintuitively inert to
235 the growth rate, and does not affect the lag phase (Figure 2). Rather, it allows the
236 bacterial community to reach a significantly higher carrying capacity that may account
237 for changes in their proportions in the GI.

238 Acid stress in Lactobacillaceae is self-imposed stress, and, thus, LAB are relatively
239 acid-tolerant, and employ several mechanisms to regulate the homeostasis of the pH
240 level (30). A. The mechanisms generally include the removal of protons or alkalization
241 of the environment via ammonia production through the arginine deiminase (ADI) (31).
242 In addition, glutamic acid decarboxylase (GAD) catalyzes the decarboxylation of
243 glutamate into gamma-aminobutyric acid (GABA), which results in the alkalization of
244 the cytoplasmic pH due to the removal of protons (32). Lastly, the urease system
245 allows the hydrolysis of urea, which enhances the survival of LAB under acid stress
246 conditions by the production of NH_3. The urease operon was found to be positively
247 regulated under low pH levels in the LAB *Streptococcus salivarius* (33). F-ATPase
248 system is another mechanism that protects LAB from acid damage. Overall, probiotic
249 acid-tolerant species are of great value as additional encapsulation in order to ensure
250 survival to transit through the acidic stomach. As the survival of LAB during acid stress
251 is quite heterogeneous the expression of these systems during self-imposed acid
252 stress and, in the stomach, needs to be properly evaluated while selecting formulating
253 probiotics. Alternatively, food carriers with high buffering potential to aid gastric transit
254 and ensure that viable cells reach the small intestine may contribute to assure a
255 beneficial effect on the host. For example, our results indicate that *L. paracasei* in
256 liquid (Figure 1) and *L. acidophilus* (Figure 4) grown on a solid medium exhibit poor

257 acid tolerance during self-imposed acid stress. One immediate application of our
258 findings for *L. paracasei* in solution (Figure 1) and *L. acidophilus* (Figure 4C) is that
259 probiotic performance of these strains in the gut is dependent in the availability of a
260 suitable buffering system.

261 In the gut, acid-sensitive species are protected from the acidic pH of the stomach (2-
262 4) because of the buffering properties of food, which depends on the type of food and
263 its volume (34, 35). In parallel, self-imposed acid stress from accumulation of organic
264 acids following the utilization of carbohydrates may affect the performance of the
265 ingested bacterial species. Interestingly, buffering of the colonies restored glucose-
266 free morphology of all strains, indicating that acid stress is involved in biofilm formation
267 throughout the Firmicutes phylum directly or indirectly. The addition of a buffering
268 system to the growth media of bacteria grown in the presence of glucose increased
269 the survival of most species significantly. However, while cell death was decreased
270 with buffering, it was still significantly higher with glucose compared with non-glucose
271 conditions (Figure 4C). Thus, the glucose-induced change in colony morphology may
272 be reflective of a complex adaptation and biofilm regulation, rather than a simple
273 reflection of cell density. While glucose utilization capacities and medium acidification
274 under our conditions were similar, the enhancement of carrying capacity (Figure 2),
275 carbohydrate mediated cell death within the colony (Figure 4C), and the adaptation to
276 glucose utilization at the single-cell level (Figure 5) varied dramatically between the
277 tested species. Changes in the surface proteome(14), quorum sensing(36), and the
278 activation of differential target genes of the stressosome (37) may differ in a context-
279 dependent manner between LAB strains. While the exact regulations remain to be
280 determined, our results indicate that the overall physiological response to
281 carbohydrate metabolism and self-imposed acid stress is not a mare reflection of
282 microbial growth.

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Materials and methods

Strains, Media and imaging

Lactobacillus acidophilus ATCC 4356, *Lacticaseibacillus casei* ATCC 393, *Lacticaseibacillus casei subsp. paracasei* ATCC BAA-52, *Lactiplantibacillus plantarum* ATCC 8014 and *Lacticaseibacillus rhamnosus GG* ATCC probiotic strains, were used in the study. *Bacillus coagulans* ATCC 10545 was used as a control. A single colony of Lactobacillaceae were isolated on a solid deMan, Rogosa, Sharpe Agar (MRS) plate, was inoculated into 5ml MRS broth (Difco, Le Pont de Claix, France), and grown at 37°C, without shaking, for overnight. A single colony of *Bacillus coagulans* isolated on a solid LB agar plate was inoculated into 5ml LB broth (Difco) and grown at 37°C, with shaking overnight. For biofilm colonies, these cultures were inoculated into a solid medium (1.5% agar) contains 50% Tryptic soy broth (TSB), TSB supplemented with (1% w/v) D-(+) - glucose, (1% w/v) D-(+)- raffinose or (1% w/v) D-(+)- mannose or (1% w/v) D-(+)-Xylose or with (1% w/v) D-(+) - glucose buffered with MOPS and potassium phosphate buffer. The bacteria were incubated in a BD GasPak™ EZ - Incubation Container with BD GasPak EZ CO2 Container System Sachets (260679) (Becton, Sparks, MD, USA), for 72 hours in 37°C. All images were taken using a Stereo Discovery V20" microscope (Tochigi, Japan) with objectives Plan Apo S ×1.0 FWD 60 mm (Zeiss, Goettingen, Germany) attached to a high-resolution microscopy Axiocam camera. Data were created and processed using Axiovision suite software (Zeiss). For planktonic growth, the bacterial cultures were inoculated into a liquid medium 50% Tryptic soy broth (TSB) with different sugars as described above, incubated for 24h, at 37°C.

Growth measurement and analysis

Cultured cells grown for overnight were diluted 1:100 in 200 µl liquid medium contains 50% Tryptic soy broth (TSB) (BD), TSB supplemented with (1% w/v) D-(+)- glucose, (1% w/v) D-(+)- raffinose or (1% w/v) D-(+)- mannose or or (1% w/v) D-(+)-Xylose in a 96-well microplate (Thermo Scientific, Roskilde, Denmark). Cells were grown with agitation at 37 °C for 18 h in a microplate reader (Tecan (Infinite® 200 PRO, Switzerland), and the optical density at 600 nm (OD_{600}) was measured every 30 min.

320 Maximum OD, growth rate and lag time calculation were performed with GrowthRates
321 3.0 software.

322

323 **Fluorescence microscopy**

324 A bacterial colony grown as described above was suspended in 200 µl 1x Phosphate-
325 Buffered Saline (PBS), and dispersed by pipetting. Samples were centrifuged briefly,
326 pelleted and re-suspended in 5µl of 1x PBS supplemented with the membrane stain
327 FM1-43 (Molecular Probes, Eugene, OR, USA) at 1 µg/ml. This cells were placed on
328 a microscope slide and covered with a poly-L-Lysine (Sigma) treated coverslip. The
329 cells were observed by Axio microscope (Zeiss, Germany). Images was analyzed by
330 Zen-10 software (Zeiss).

331

332 **pH measurements**

333 After inoculation in different liquid mediums and 24h incubation (without shaking) at
334 37°C, the cells were separated from the medium by centrifugation (4000xg, 20 min)
335 followed by filtration through a 0.22 µm. The conditioned media acidity was measured
336 by the pH meter (Mettler Toledo). pH measurements of the solid media were done
337 using pH-indicator strips (MQuant®, Merck).

338

339 **Determination of organic acids**

340 The supernatant of the bacteria grown on MSgg with 1% glucose for 24h was filtered
341 through 0.22 µm filter membranes for HPLC analysis. The contents of organic acids in
342 each liquid sample were determined using an Infinity 1260 (Agilant Technologies)
343 HPLC system with C 18 column (Syncronis™ C18 Columns, 4.6 × 250 mm, 5 µm).
344 Mobile phase A was acetonitrile and mobile phase B was 5-mM KH_2PO_4 pH 2.4. The
345 flow rate was kept constant at 1 mL/min, with ultraviolet detection performed at
346 210 nm. The injection volume was 20 µL and the column temperature was maintained
347 at 30 °C. Identification and quantification of organic acids were accomplished by
348 comparing the retention times and areas with those of pure standards.

349 Notably, although quantification of organic acids in TSB was not feasible due to high
350 background, the comparable acidity strongly indicates similar fermentation capacities
351 of the strains tested in rich growth media.

352

353 **Flow Cytometry Analysis**

354 Starter cultures were spotted on TSB with glucose or TSB with glucose buffered with
355 MOPS and potassium phosphate buffer. The plates were then incubated as mentioned
356 in the first section. Colonies were harvested after 72 hours. Samples were diluted in
357 PBS and measured using an LSR-II new cytometer (Becton Dickinson, San Jose, CA,
358 USA). PI (Propidium Iodide, 20Mm, Invitrogen™) fluorescence was measured using
359 laser excitation of 488 nm, coupled with 600 LP and 610/20 sequential filters. A total
360 of 100,000 cells were counted for each sample and flow cytometry analyses was
361 performed using BD FACSDiva™ software.

362

363 **Protein alignment, phylogenetic tree**

364 Phylogenetic tree was built based on 16S of the bacteria and multiple alignment
365 sequences of LDH proteins was preformed using Clustal Omega.
366 https://www.ebi.ac.uk/Tools/msa/clustalo/

367

368 **Statistical analysis**

369 All experiments were performed at least three separate and independent times in
370 triplicates, unless stated otherwise. Statistical analyses were performed with
371 GraphPad Prism 9.0 (GraphPad 234 Software, Inc., San Diego, CA). Relevant
372 statistical test are mentioned in the indicated legends of the figures.

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484 **Legends**

485 Figure 1. **Heterogeneous alterations in planktonic growth of LAB strains during**
486 **glucose utlization.** Planktonic growth of the indicated species **(A)** LGG **(B)** *L. casei*
487 **(C)** *L. acidophilus* **(D)** *L. paracasei* **(E)** *L. plantarum* **(F)** and *B. coagulans* in TSB
488 medium (control) in TSB medium either supplemented or not with glucose (1% W/V)
489 and raffinose (1% W/V). Graphs represent mean ± SEM from six independent
490 experiments (n = 24).

491

492 Figure 2. **Carbon-dependent growth induction is specifically manifested by an**
493 **altered carrying capacity.** Analysis of planktonic growth from the data shown in
494 Figure 1 using GrowthRates 3.0. **A)** Fold Change in carrying capacity of indicated
495 species between glucose/control, raffinose/control and glucose/raffinose. **B)** Fold
496 Change in growth rate of indicated species between glucose/control, raffinose/control
497 and glucose/raffinose. **C)** Fold Change in lag time of indicated species between
498 glucose/control, raffinose/control and glucose/raffinose. Graphs represent mean ± SD
499 from six independent experiments (n = 24). Statistical analysis was performed using
500 Brown-Forsthye and Welch's ANOVA with Dunnett's T3 multiple comparisons test. $P <$
501 0.05 was considered statistically significant.

502

503 Figure 3. **The variation at the specie level in growth enhancement is not due to**
504 **glucose catabolism. A)** The pH of the conditioned media of indicated species in TSB
505 medium (control) and TSB medium supplemented with glucose (1% W/V) and
506 raffinose (1% W/V). Statistical analysis was performed using two-way ANOVA followed
507 by Dunnett's multiple comparison test. $P < 0.05$ was considered statistically significant.
508 **B)** HPLC chromatograms indicates an exclusive peak and stable retention time for the
509 pure standards of Lactic acid and Acetic acid. **C)** The concertation of lactic acid and
510 acetic acid produced by the indicated species. Bacteria were grown on MSgg medium
511 supplemented with glucose (1% W/V) for 24h. The conditioned medium from the
512 cultures was collected and analyzed using C-18 column. Statistical analysis was
513 performed using Brown-Forsthye and Welch's ANOVA with Dunnett's T3 multiple
514 comparisons test. $P < 0.05$ was considered statistically significant.

515

516 Figure 4. **Self-imposed acidic stress has a role in shaping microbial colonies. A)**
517 Shown are the indicated biofilm colonies grown on TSB medium (control), TSB
518 medium supplemented with glucose and raffinose (1% W/V), and TSB medium
519 supplemented with glucose (1% W/V) + buffer. Biofilms were grown at 37° C in in CO_2
520 enriched environment . Biofilms colonies were imaged at 72 hours post inoculation.
521 Scale bar = 2mm. **B)** Measurement of pH of the indicated species as shown in A.
522 Statistical analysis was performed using two-way ANOVA followed by Dunnett's
523 multiple comparison test. $P < 0.05$ was considered statistically significant. **C)** Flow
524 cytometry analysis of number of dead cells of the indicated species as shown in A.
525 Colonies were grown on TSB medium supplemented with glucose (1% W/V), and TSB
526 medium supplemented with glucose (1% W/V) + buffer. Data were collected from 72
527 hours post inoculation, 100,000 cells were counted. Y-axis represents the % of dead
528 cells, graphs represent mean ± SD from three independent experiments (n = 9).
529 Statistical analysis was performed between glucose and glucose + buffer using
530 unpaired two tailed t test with Welch's correction. $P < 0.05$ was considered statistically
531 significant. **D)** Table showing multiple comparison test between dead cell populations
532 of indicated species grown in TSB medium supplemented with glucose (1% W/V).
533 Statistical analysis was performed using Brown-Forsthye and Welch's ANOVA with
534 Dunnett's T3 multiple comparisons test. $P < 0.05$ was considered statistically
535 significant.

536

537 Figure 5. **Self-imposed acidic stress induces a polymorphism in cellular**
538 **arrangements of colony cells.** Shown are the indicated fluorescence microscope
539 images of cells from biofilm colonies that were grown on TSB medium (control), TSB
540 medium supplemented with glucose and raffinose (1% W/V), and TSB medium
541 supplemented with glucose (1% W/V) + buffer. Cells were stained using Green-
542 membrane stain FM™ 1-43FX. Biofilms were grown at 37° C in CO_2 enriched
543 environment. Cells were imaged at 72 hours post inoculation. The images represent
544 3 independent experiment, from each repeat at least 10 fields examined. Scale bar =
545 2μm.

546

547

Figure 1

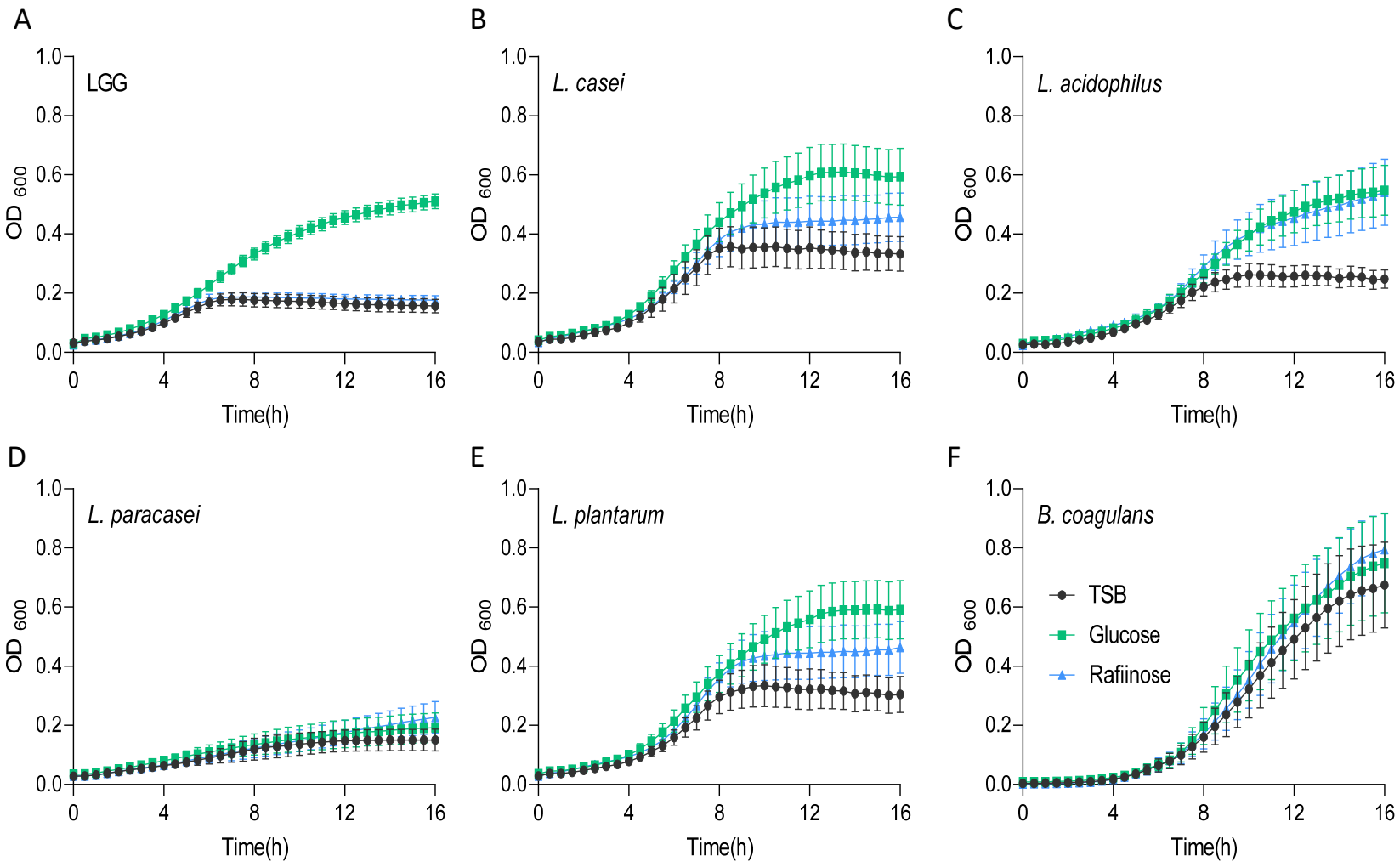

A
LGG
B
L. casei
C
L. acidophilus
D
L. paracasei
E
L. plantarum
F
B. coagulans
TSB
Glucose
Rafiinose
OD 600
Time(h)
0.0
0.2
0.4
0.6
0.8
1.0
0
4
8
12
16

Figure 2

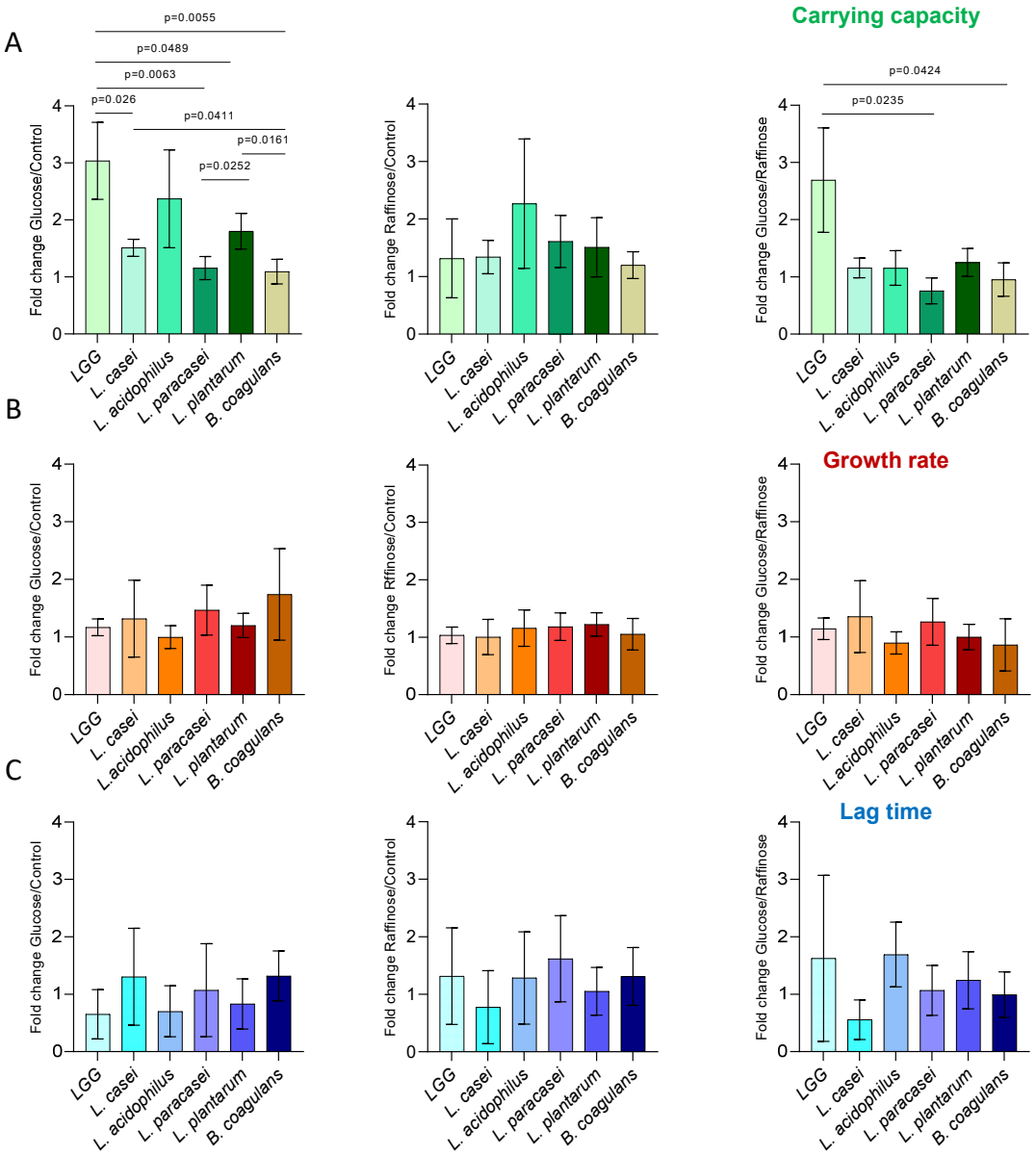

A
B
C
Carrying capacity
Growth rate
Lag time
p=0.0055
p=0.0489
p=0.0063
p=0.026
p=0.0411
p=0.0161
p=0.0252
p=0.0424
p=0.0235
Fold change Glucose/Control
Fold change Raffinose/Control
Fold change Glucose/Raffinose
Fold change Rffinose/Control
Fold change Glucose/Raffinose
LGG
L. casei
L. acidophilus
L. paracasei
L. plantarum
B. coagulans

Figure 3

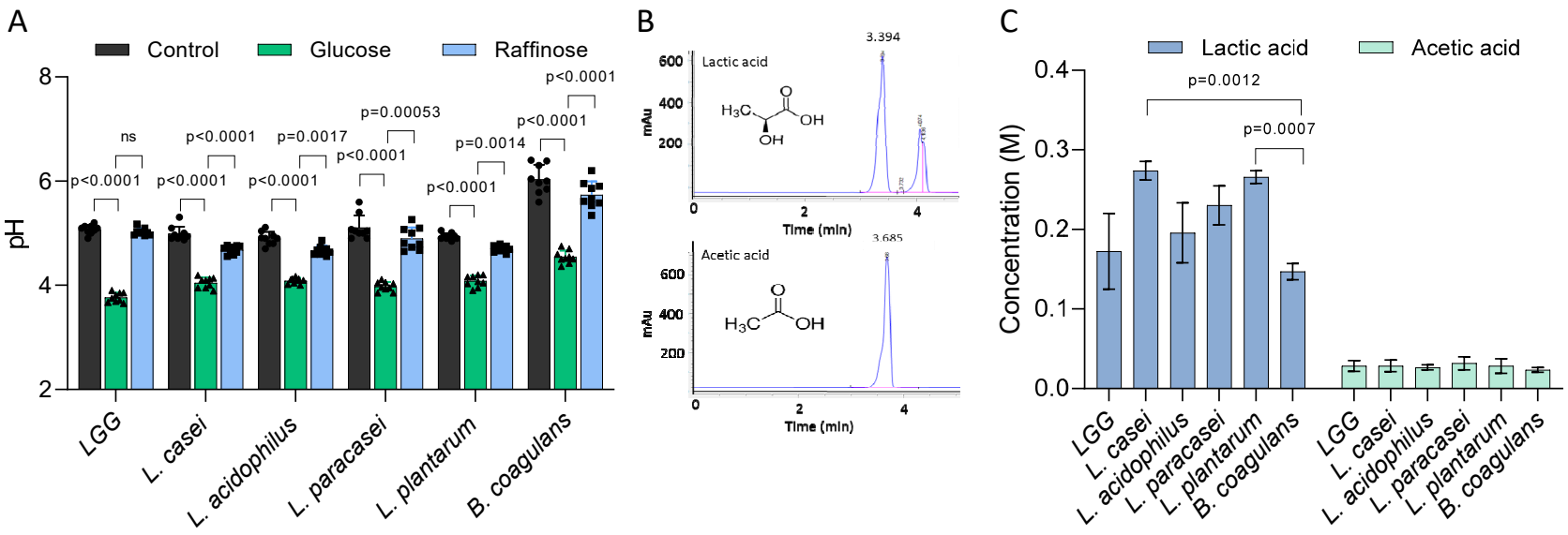
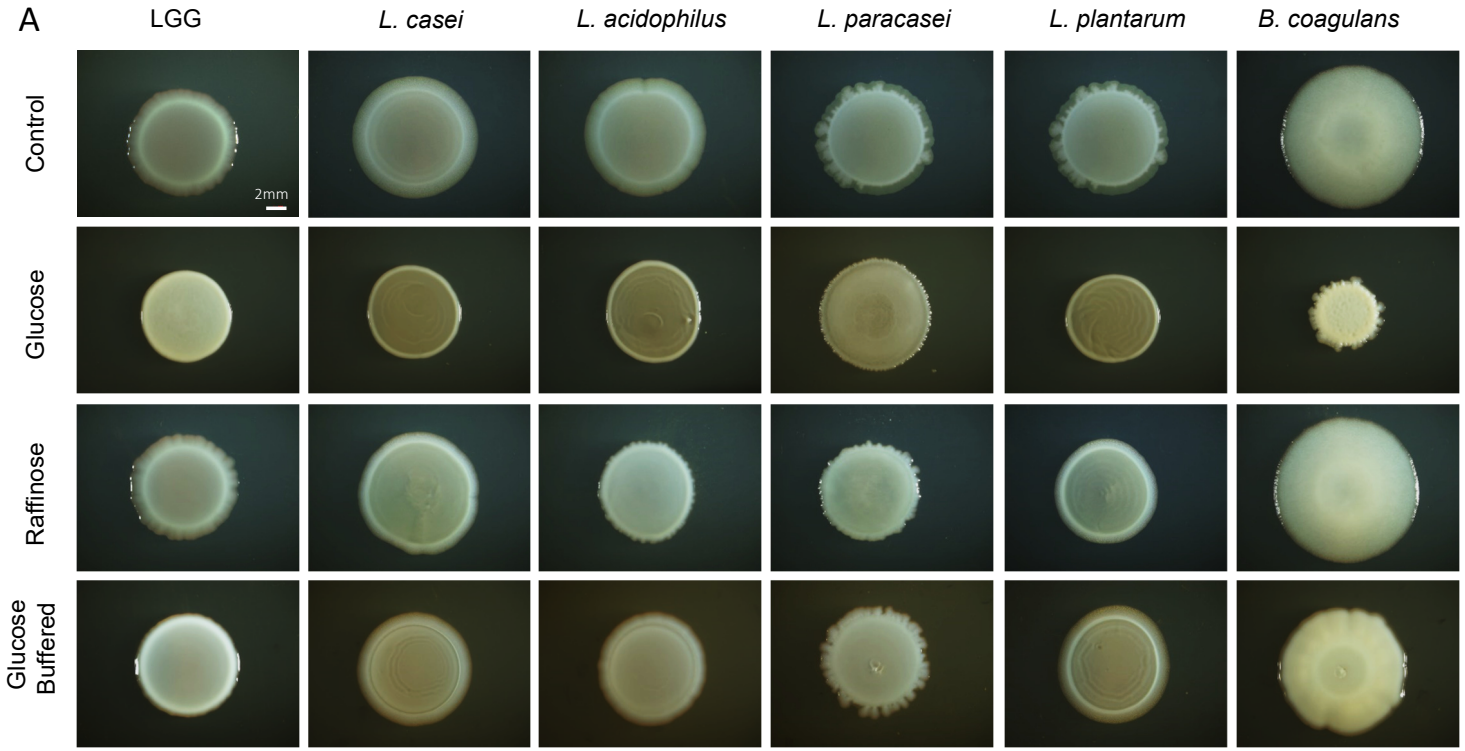
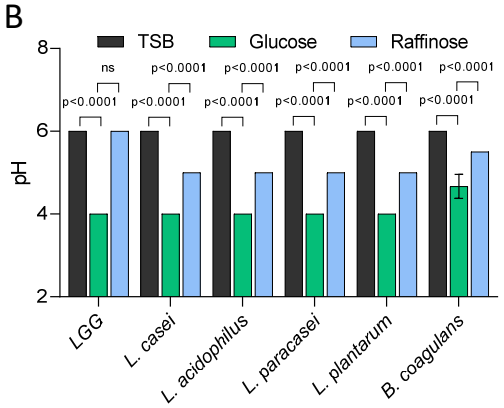
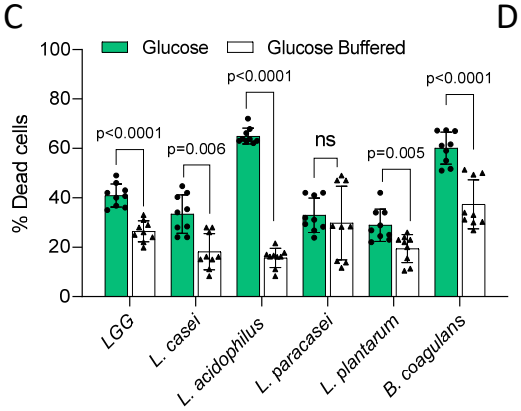

A
Control
Glucose
Raffinose
pH
8
6
4
2
ns
p<0.0001
p<0.0001
p<0.0001
p<0.0001
p=0.0017
p<0.0001
p=0.00053
p<0.0001
p=0.0014
p<0.0001
p<0.0001
p<0.0001
LGG
L. casei
L. acidophilus
L. paracasei
L. plantarum
B. coagulans
B
Lactic acid
3.394
mAu
600
400
200
0
2
4
Time (min)
Acetic acid
3.685
C
Lactic acid
Acetic acid
Concentration (M)
0.4
0.3
0.2
0.1
0.0
p=0.0012
p=0.0007
LGG
L. casei
L. acidophilus
L. paracasei
L. plantarum
B. coagulans

Figure 4



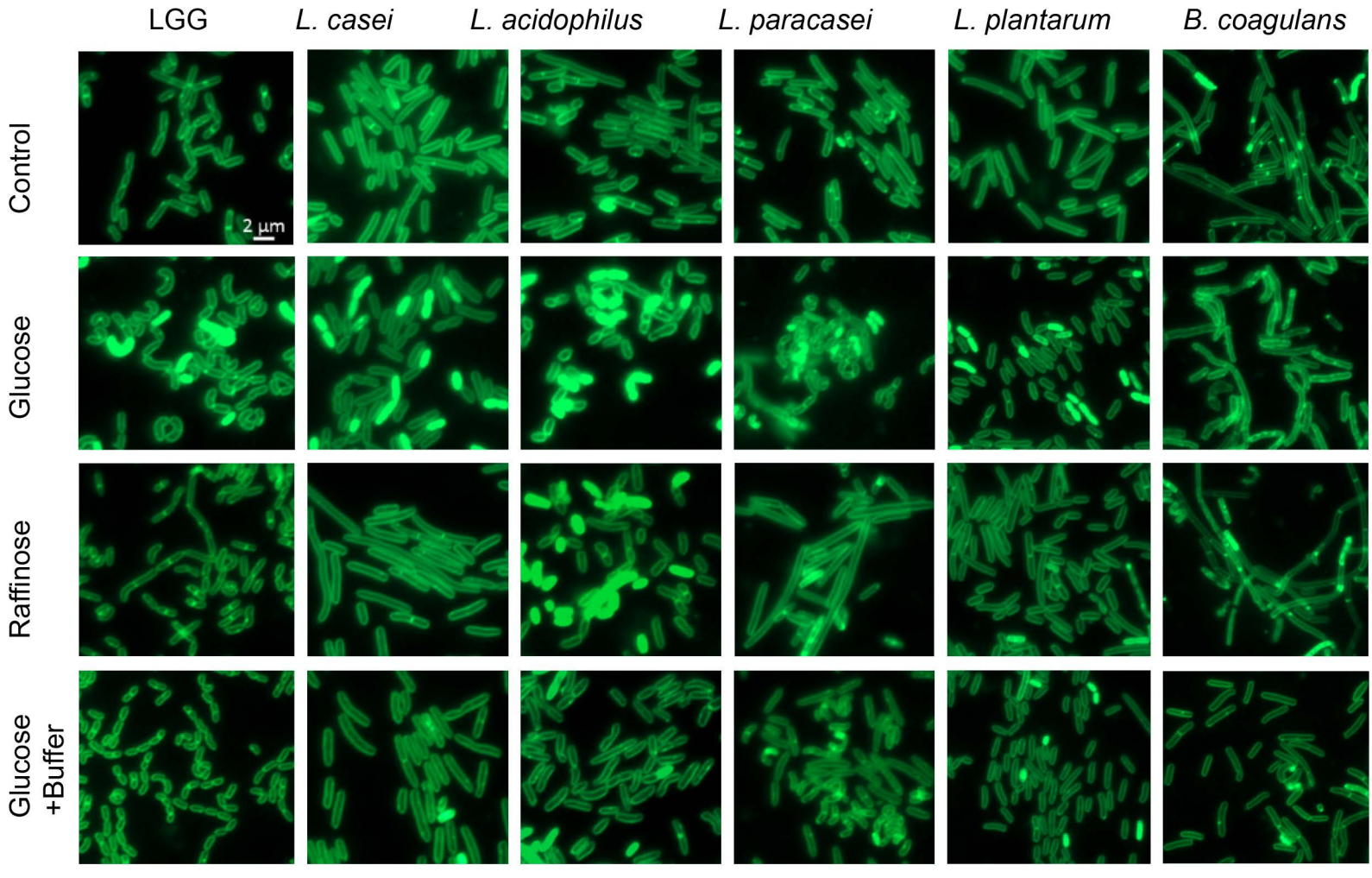




D

	P Value
LGG vs. L. acidophilus	*<0.0001*
LGG vs. L. plantarum	*0.0071*
LGG vs. B. coagulans	*<0.0001*
L. casei vs. L. acidophilus	*<0.0001*
L. casei vs. B. coagulans	*<0.0001*
L. acidophilus vs. L. plantarum	*<0.0001*
L. plantarum vs. B. coagulans	*<0.0001*

Figure 5


LGG
L. casei
L. acidophilus
L. paracasei
L. plantarum
B. coagulans
Control
Glucose
Raffinose
Glucose +Buffer
2 µm

Context-dependent differences in the functional responses of Lactobacillaeca strains to fermentable sugars

Ronit Suissa[1,2], Rela Oved[1], Harsh Maan[1], Omri Gilhar[1], Michael Meijler[2], Omry Koren[3] and Ilana Kolodkin-Gal[1,4@]

Supporting Information

Supporting Figures (S1-S3)

Supporting Table (S1)

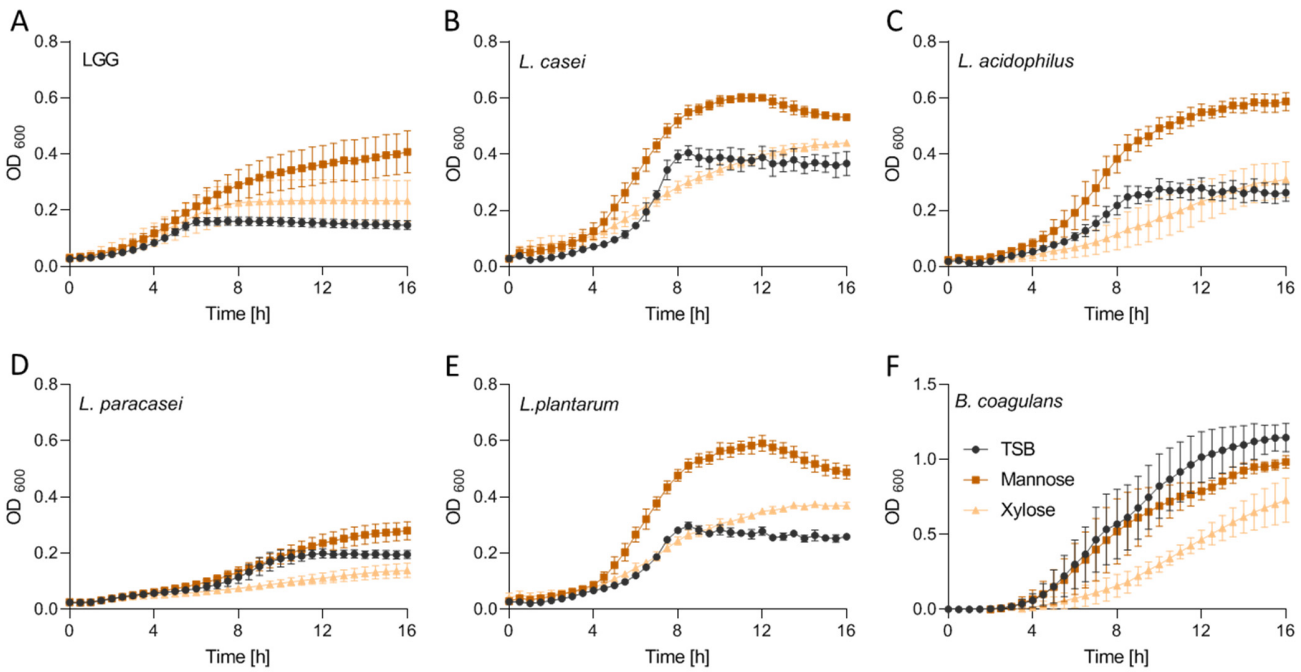


Figure S1. **The effect of fermentable sugars on planktonic growth.** Planktonic growth of the indicated species (A) LGG (B) *L. casei* (C) *L. acidophilus* (D) *L. paracasei* (E) *L. plantarum* (F) and *B. coagulans* in TSB medium (control) and TSB medium supplemented with mannose (1% W/V) and xylose (1% W/V). Graphs represent mean ± SD from three independent experiments (n =3).

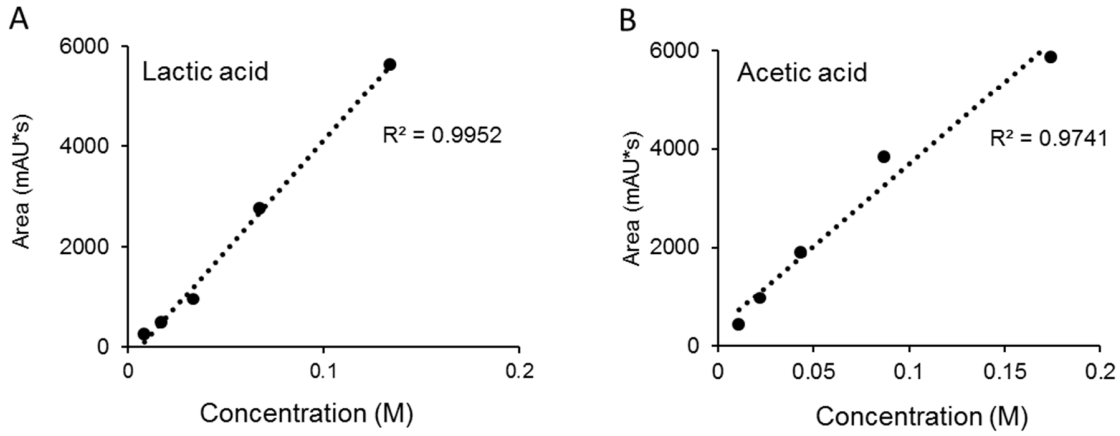


Figure S2. **Calibration curves** for lactic acid (A) and acetic acid (B)

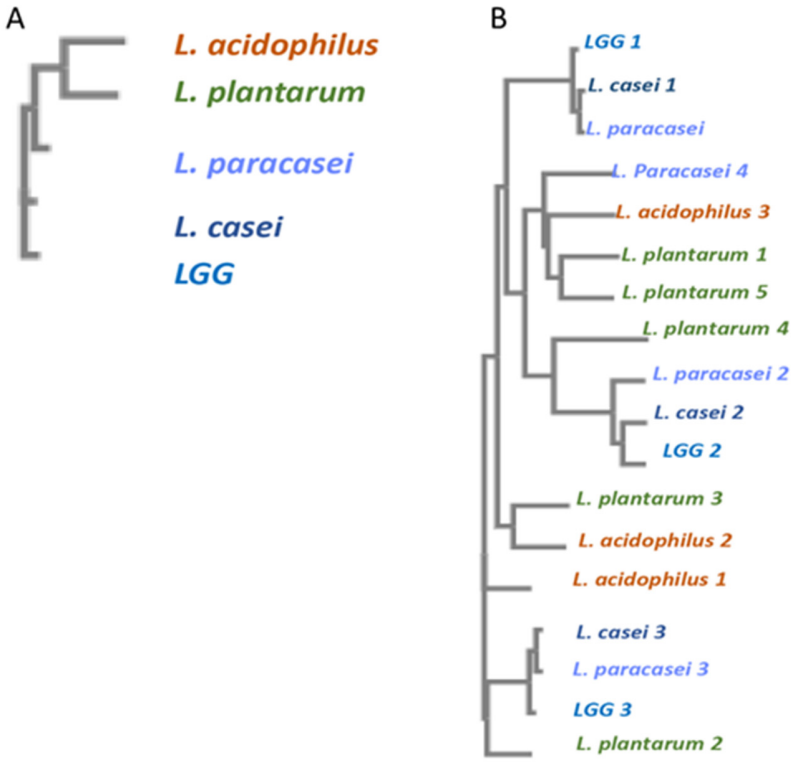


Figure S3. **In silico analysis of LDH proteins**. Phylogenetic trees were generated using Clustal Omega based on A)16S or B) L- LDH proteins

	TSB	TSB	TSB	Glucose	Glucose	Glucose	Raffinose	Raffinose	Raffinose
LGG	6	6	6	4	4	4	6	6	6
L. casei	6	6	6	4	4	4	5	5	5
L. acidophilus	6	6	6	4	4	4	5	5	5
L. paracasei	6	6	6	4	4	4	5	5	5
L. plantarum	6	6	6	4	4	4	5	5	5
B.coagulans	6	6	6	4.5	4.5	5	5.5	5.5	5.5

Table S1. Table shows the individual values of pH measurements indicated in figure 4B from three independent experiments.