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How carbon sources drive cellulose synthesis in two *Komagataeibacter xylinus* strains

Federico Lasagni, Stefano Cassanelli ✉ & Maria Gullo ✉

Bacterial cellulose synthesis from defined media and waste products has attracted increasing interest in the circular economy context for sustainable productions. In this study, a glucose dehydrogenase-deficient Δ*gdh* K2G30 strain of *Komagataeibacter xylinus* was obtained from the parental *wild type* through homologous recombination. Both strains were grown in defined substrates and cheese whey as an agri-food waste to assess the effect of gene silencing on bacterial cellulose synthesis and carbon source metabolism. *Wild type* K2G30 boasted higher bacterial cellulose yields when grown in ethanol-based medium and cheese whey, although showing an overall higher D-gluconic acid synthesis. Conversely, the mutant Δ*gdh* strain preferred D-fructose, D-mannitol, and glycerol to boost bacterial cellulose production, while displaying higher substrate consumption rates and a lower D-gluconic acid synthesis. This study provides an in-depth investigation of two *K. xylinus* strains, unravelling their suitability for scale-up BC production.

Keywords *Komagataeibacter xylinus*, Glucose dehydrogenase, Bacterial cellulose, Gluconic acid, Agro-wastes

Developing sustainable processes and products by using microbial resources encompasses different strategies and tools aimed at obtaining added value commodities while reducing pollution. Among biopolymers, bacterial cellulose (BC) has been extensively studied, together with the organisms responsible for its production. Moreover, several studies demonstrated the feasibility of using wastes to synthesize BC through selective fermentations. Nowadays, agri-food wastes represent one of the most alarming environmental and social threats, and its importance is constantly growing due to the increase in global food demand[1]. The Food and Agriculture Organization of the United Nations (FAO) estimated an average of 570 million tonnes of wasted food yearly worldwide only by household habits[2], leading to massive resource squandering, air pollution, and spread undernutrition[3]. In the perspective of a circular economy, many processes have been perfected to exploit agri-food wastes and byproducts, such as biofuel production[4] and energy recovery[5]. Furthermore, a microbial approach has laid the foundations for novel biotechnological strategies, paving the way for the sustainable synthesis of biopolymers[6]. BC synthesis has gained increasing interest in this context as a promising processing tool for food industry wastes[7], mainly thanks to the high BC-producing acetic acid bacterium *Komagataeibacter xylinus*[8,9]. Within the circular economy framework, selecting BC-producing strains for the biological synthesis of BC from pure carbon sources and agro-wastes can contribute to address environmental challenges, thus providing a sustainable approach for BC production.

In this study, we hypothesized that BC synthesis in *K. xylinus* is highly affected by growth substrate properties. Different carbon sources may trigger switches in metabolic pathways, modulating the cell's phenotypic response. As a result, culture conditions may turn into an unsuitable environment for BC synthesis, primarily due to high D-gluconic acid (GlcAc) production. To address this issue, a parental *wild type* K2G30 strain and a mutant (Δ*gdh*), here obtained by silencing the gene encoding for the membrane-bound, pyrroloquinoline quinone-dependent glucose dehydrogenase (PQQ-GDH), were tested (Fig. 1). Both strains were assessed for BC synthesis in several defined media and cheese whey (CW), monitoring their respective carbon source consumption and GlcAc production over cultivation times. Our results demonstrate a variability in BC synthesis between *wild type* and Δ*gdh* K2G30 strains depending on the growth medium. Moreover, a different substrate uptake was observed and correlated with the oxidative pathway leading to GlcAc production.

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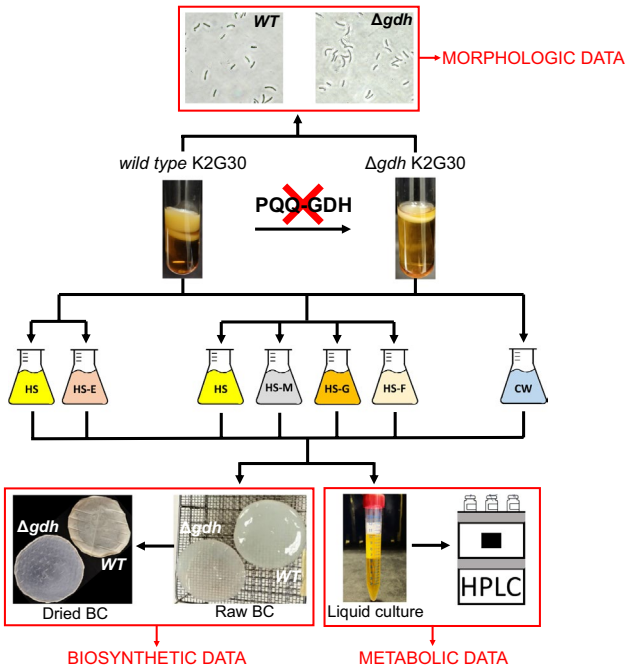

Fig. 1. Schematic representation of the work strategy. A mutant Δ*gdh* K2G30 strain was obtained from *wild type* K2G30 through PQQ-GDH suppression. After a brief morphological characterization (morphologic data), both strains were grown in different HS media and cheese whey (CW) to assess bacterial cellulose (BC) synthesis (biosynthetic data), in parallel with the respective consumption of each carbon source and D-gluconic acid production (metabolic data).

Results

Δ*gdh* K2G30 genotype assessment

The PCR screening confirmed the correct homologous integration of AMPr cassette into the *gdh* gene (Fig. S1a). PCR encompassing the target gene showed the expected increase of amplicon size in Δ*gdh* K2G30 compared to *wild type* K2G30, while amplifications of both the 5' and 3' ends of the construct insertions only occurred in Δ*gdh* K2G30 (Fig. S1b). Finally, direct sequencing of gdh-R2/AMPr-F2 PCR product validated the accuracy of AMPr cassette insertion in Δgdh K2G30 (Fig. S2). The proper functioning of the AMPr cassette was confirmed by the ability of Δ*gdh* K2G30 to grow on HS-A-Amp forming small, spheroidal, point-like, non-pigmented defined colonies, according to Prudnikova and Shidlovsky[10] (Fig. S3b). Conversely, *wild type* K2G30 strain did not grow on HS-A-Amp medium (Fig. S3c), while highlighting a rich BC synthesis in HS-A similar to Δ*gdh* K2G30 (Fig. S3a).

Bacterial growth and morphology

Wild type and Δ*gdh* K2G30 strains showed different phenotypic behaviours during the revitalization period in HS (Fig. 2). The *wild type* culture formed a thin exopolysaccharide-like layer at the liquid–air interface after 48 h of growth, which evolved in a thin biofilm pellicle on the fourth day (Fig. 2a). Conversely, the mutant Δ*gdh* strain did not show any biofilm production until the third day of incubation (Fig. 2b). As growth progressed, the thickness of the biofilm pellicles increased significantly. According to the characterization method described by Navarro and Komagata[11], the biofilm material synthesized by both strains showed properties addressable to BC, as confirmed by its insolubility after boiling in NaOH 5% w/v[11]. No morphological differences were observed between strains, which shared rod-shaped cells proper of *Komagataeibacter* genus-belonging bacteria (Fig. S4).

Ethanol enhances bacterial cellulose synthesis differently in *K. xylinus* strains

Both strains depleted ethanol (7.89 g/L) within the first days of growth, although showing different metabolic behaviours. On the sixth day of incubation, Δ*gdh* K2G30 stood out for the maximum BC yield (7.947 ± 0.618 g/L) in HS medium, while BC synthesis in HS-E was far lower (2.927 ± 0.481 g/L). Conversely, the BC yield obtained by *wild type* K2G30 in HS-E (6.060 ± 0.191 g/L) was greater than in HS (2.600 ± 0.420 g/L) (Fig. 3). D-glucose uptake between strains showed no significant differences, except for the lowest consumption detected for Δ*gdh* K2G30 in HS-E (6.419 ± 0.349 g/L). As growth progressed, an increased BC yield was displayed by Δ*gdh* K2G30 in the same medium (9.400 ± 1.002 g/L), although a major BC synthesis was carried out in HS (12.260 ± 0.926 g/L). The results are consistent with the highest D-glucose uptake detected for Δ*gdh* K2G30 in HS (19.004 ± 0.259 g/L), while other culture conditions did not show significant differences on the eleventh day of incubation (Fig. 4). A more detailed analysis revealed a general increase in BC synthesis among the time points, highlighting the maximum increment (+ 221%) for Δ*gdh* K2G30 in HS-E. The trend of BC synthesis showed a delayed production

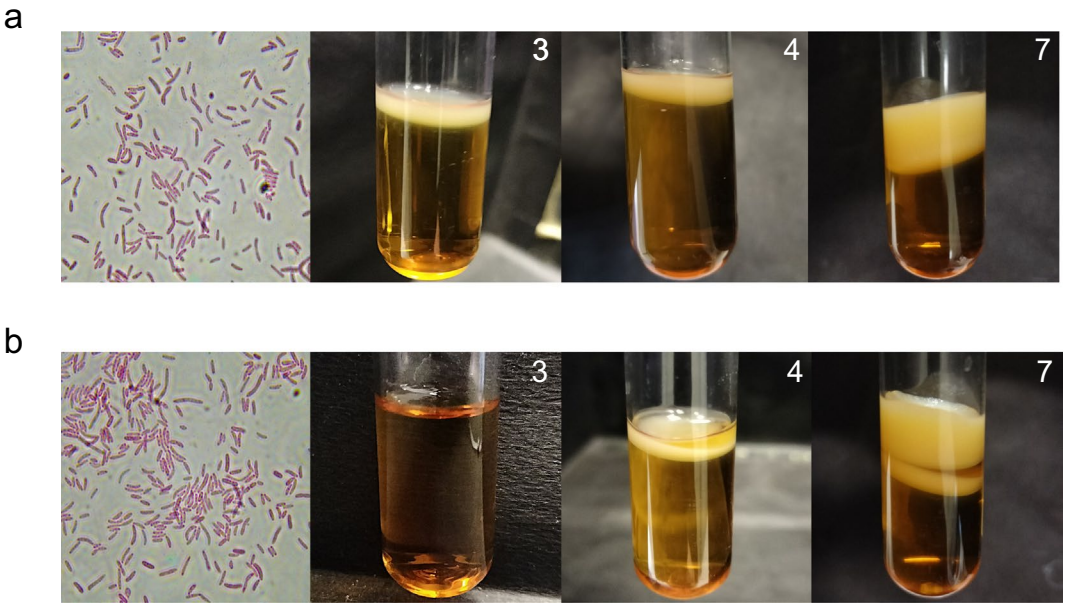

Fig. 2. *Wild type* K2G30 (**a**) and Δ*gdh* K2G30 (**b**) strains: gram staining at seven days of cultivation in HS medium and BC synthesis during the revitalisation period (days 3, 4, 7), respectively.

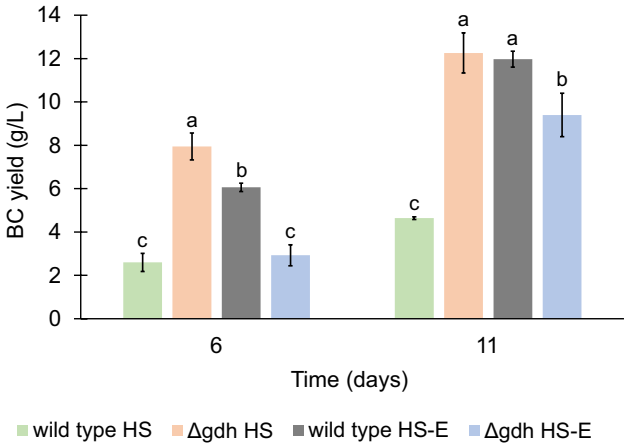

Fig. 3. Bacterial cellulose (BC) synthesis by *wild type* and Δ*gdh* K2G30 strains after six and eleven days of growth in HS and HS-E media. Experimental data are shown as mean ± standard deviation among technical triplicates (n = 3). Different letters highlight significant differences ($p < 0.05$) between strains in the same medium.

by Δ*gdh* K2G30; this behaviour was accompanied by a slow glucose uptake by the mutant strain (Fig. 4), according to literature[12,13]. BC synthesis in both strains was coupled with a significant GlcAc production (Fig. 5). *Wild type* K2G30 displayed the highest GlcAc yield on the sixth day, although no significant differences were detected between HS (8.533 ± 0.760 g/L) and HS-E (7.458 ± 0.445 g/L) media (Fig. 5a). As growth progressed, a lower GlcAc synthesis equals to 3.720 ± 0.130 g/L and 3.162 ± 0.140 g/L was achieved by the same strain grown in HS and HS-E media, respectively (Fig. 5b). Contrary to *wild type*, Δ*gdh* K2G30 showed a poor GlcAc synthesis on the sixth day in HS (1.465 ± 0.015 g/L) and HS-E (1.365 ± 0.038 g/L) media. An increase in GlcAc synthesis by the mutant Δ*gdh* strain was therefore detected on the eleventh day in both HS (2.348 ± 0.021 g/L) and HS-E (2.558 ± 0.096 g/L) media.

Bacterial cellulose and gluconic acid synthesis are strongly affected by different carbon sources

The influence of different substrates on BC synthesis revealed a divergent behaviour between strains. On the fourth day, no significant differences were detected among BC yields produced by strains grown in HS media, except for HS-M, where *wild type* K2G30 displayed a higher BC yield (1.771 ± 0.110 g/L) than Δ*gdh* K2G30 (0.831 ± 0.062 g/L). A negligible BC synthesis was also observed in HS-G (Fig. 6a), where both strains

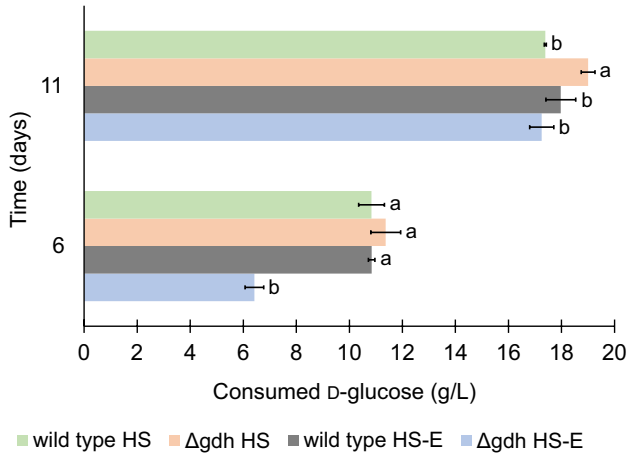

Fig. 4. D-glucose consumed by *wild type* and Δ*gdh* K2G30 strains after six and eleven days of growth in HS and HS-E media. Experimental data are shown as mean ± standard deviation among technical triplicates (n = 3). Different letters highlight significant differences ($p < 0.05$) between strains at the same time set point.

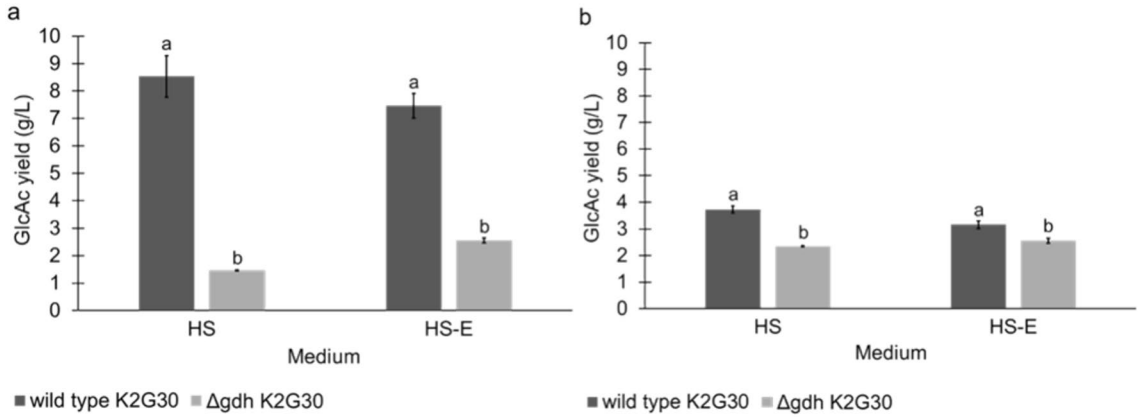

Fig. 5. D-gluconic acid (GlcAc) yield produced by *wild type* and Δ*gdh* K2G30 strains after six (**a**) and eleven (**b**) days of growth in HS and HS-E media. Experimental data are shown as mean ± standard deviation among technical triplicates (n = 3). Different letters highlight significant differences ($p < 0.05$) between strains in the same medium.

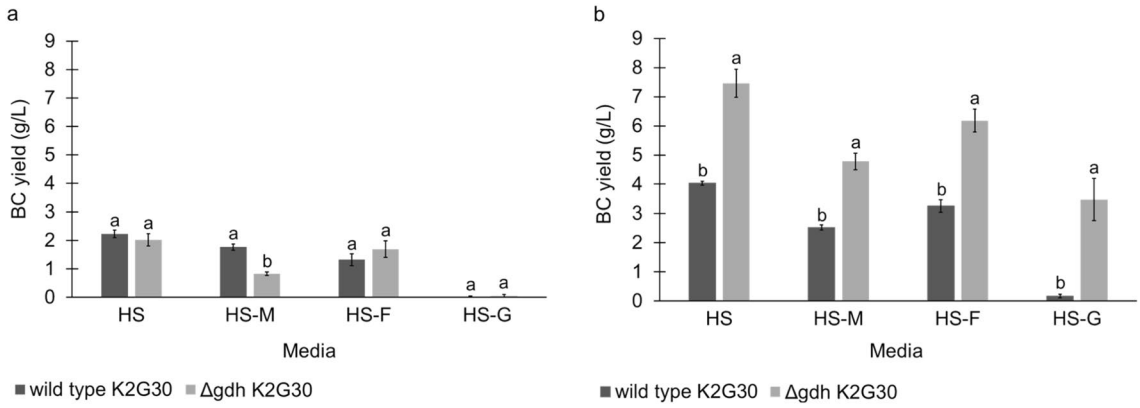

Fig. 6. Bacterial cellulose (BC) yield produced by *wild type* and Δ*gdh* K2G30 strains after four (**a**) and seven (**b**) days of growth in various carbon sources-containing media: D-glucose (HS), D-mannitol (HS-M), D-fructose (HS-F) and glycerol (HS-G). Experimental data are shown as mean ± standard deviation among technical triplicates (n = 3). Different letters highlight significant differences ($p < 0.05$) between strains in the same medium.

produced a submerged, jelly-like mass instead of a defined BC layer. Conversely, at the end of the incubation period, Δ*gdh* K2G30 stood out for a higher BC yield in every culture condition (Fig. 6b), showing a peak in HS (7.466 ± 0.482 g/L) and a major yield increment is HS-G. Carbon source consumption analysis revealed a higher D-glucose uptake for *wild type* K2G30 (6.893 ± 0.476 g/L) on the fourth day, as for D-mannitol (4.280 ± 0.297 g/L), while Δ*gdh* K2G30 seemed to prefer D-fructose (6.032 ± 0.121 g/L) (Fig. 7a); no significant differences in glycerol uptake were detected between strains. At the end of the incubation period, Δ*gdh* K2G30 stood out for a higher D-mannitol (11.893 ± 0.368 g/L), D-fructose (9.535 ± 0.458 g/L) and glycerol (8.809 ± 0.625 g/L) uptake, although *wild type* K2G30 still showed the maximum D-glucose consumption (12.516 ± 0.094 g/L) (Fig. 7b). On the seventh day of incubation, *wild type* K2G30 stood out for the highest GlcAc synthesis achieved in HS medium (6.070 ± 0.098 g/L); likewise, the yield produced by Δ*gdh* K2G30 in the same condition was far lower (1.348 ± 0.107 g/L). Other carbon sources did not stimulate GlcAc synthesis as D-glucose did (Fig. 8).

Cheese whey is a suitable substrate for bacterial cellulose synthesis

In this study, CW was selected among other food byproducts according to its compliance with specific requirements and consolidated applications[7,14]. The carbon source content of raw CW is reported in Table S7. The microbial growth in CW elicited divergent responses between strains, which can be attributed to variations in their metabolic patterns and the complexity of the substrate. *Wild type* K2G30 showed a higher BC yield than the mutant Δ*gdh* strain at every time point, reaching a peak (12.891 ± 0.161 g/L) on the eleventh day of incubation. Conversely, Δ*gdh* K2G30 stood out for the maximum BC increment between the two latest time points (Fig. 9). When grown in CW, *wild type* K2G30 produced an overall higher BC yield after four and seven days compared

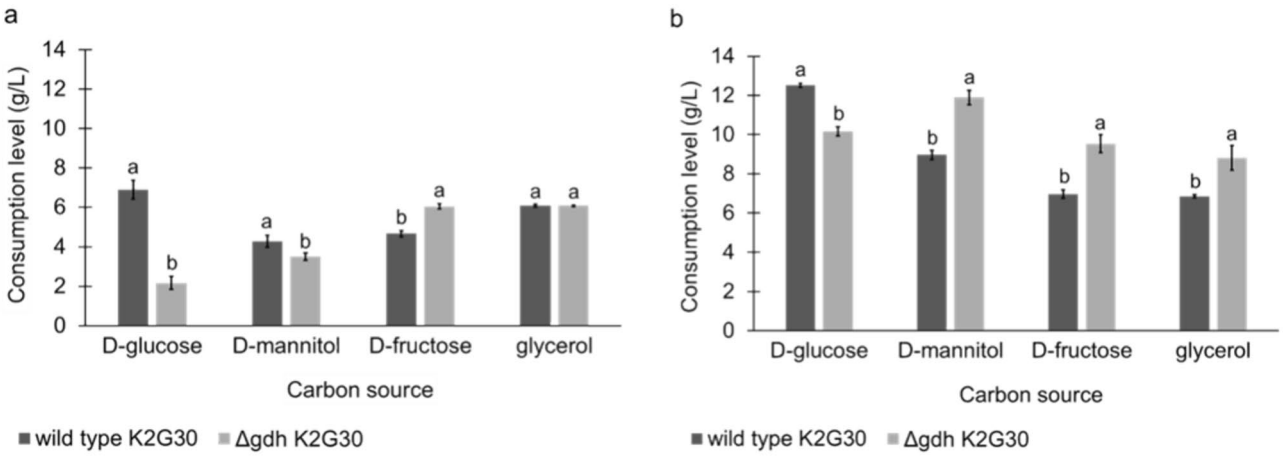

Fig. 7. Consumption of D-glucose, D-mannitol, D-fructose, and glycerol by *wild type* and Δ*gdh* K2G30 strains grown on different HS media after four (**a**) and seven (**b**) days of incubation. Experimental data are shown as mean ± standard deviation between technical triplicates (n = 3). Different letters highlight significant differences ($p < 0.05$) in consuming the same carbon source between strains.

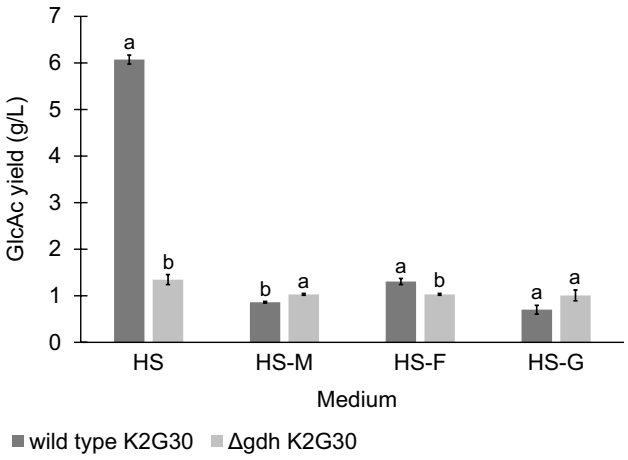

Fig. 8. D-gluconic acid (GlcAc) yield produced by *wild type* and Δ*gdh* K2G30 strains after seven days of growth in different HS media (HS, HS-M, HS-F, HS-G). Experimental data are shown as mean ± standard deviation among technical triplicates (n = 3). Different letters highlight significant differences ($p < 0.05$) between strains in the same medium.

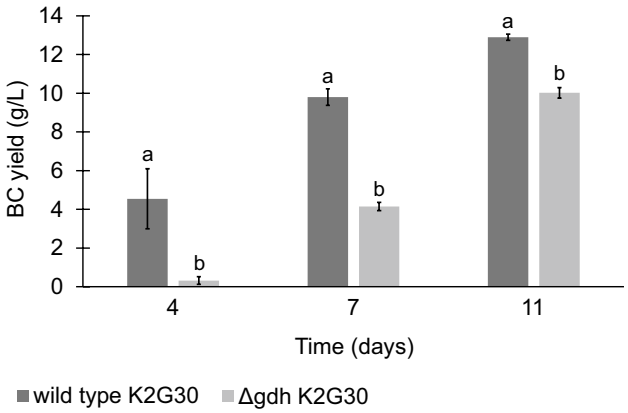

Fig. 9. Bacterial cellulose (BC) produced by *wild type* and Δ*gdh* K2G30 strains after four, seven and eleven days of growth in cheese whey (CW). Experimental data are shown as mean ± standard deviation among technical triplicates (n = 3). Different letters highlight significant differences ($p < 0.05$) between strains at the same time set point.

to HS media, contrary to Δ*gdh* K2G30 (Fig. 6). Moreover, no significant differences in D-lactic acid (LacAc) and D-lactose consumption between strains were detected until day seven (Fig. 10), where *wild type* K2G30 displayed a higher LacAc consumption (3.423 ± 0.103 g/L). At the end of the incubation period, the maximum LacAc uptake was performed by *wild type* K2G30 (4.121 ± 0.028 g/L), equal to almost 86% of the initial LacAc concentration (Fig. 10a). D-lactose consumption did not show significant differences between strains since day four (Fig. 10b), while free D-galactose was not depleted at all. Carbon sources uptake followed a sigmoidal progression during the incubation period, according to Keshk and Sameshima[15]; in addition, the highest increments in BC yield were coupled with an increase in LacAc consumption, as particularly evident for *wild type* K2G30. Alongside BC synthesis, a negligible GlcAc production was detected for *wild type* (0.935 ± 0.010 g/L) and Δ*gdh* (1.013 ± 0.051 g/L) K2G30 strains at the end of the incubation period (Fig. S5).

Discussion

K. xylinus can metabolize various carbon sources to D-glucose to support BC synthesis[16]. A large portion of D-glucose may be therefore oxidized to GlcAc by a PQQ-GDH[13]. This enzymatic activity results in a rapid decrease of pH, as well as depleting D-glucose molecules that would be otherwise available for polymerization into BC[17]. According to the literature[13,18], silencing the gene responsible for PQQ-GDH activity could potentially increase the substrate availability for BC synthesis, simultaneously decreasing GlcAc production. In *wild type* cells, after being exported in the surrounding medium, a part of GlcAc can be internalized in both Entner-Doudoroff (ED) and pentose phosphate (PP) pathways, forming pyruvate (PYR), glyceraldehyde-3-phosphate (GA3P) and fructose-6-phosphate (F6P)[19,20]. Due to the absence of a phosphofructokinase 1 (PFK1)-encoding gene[21], *K. xylinus* cannot further catabolize F6P through glycolysis, while GA3P is still available to form PYR

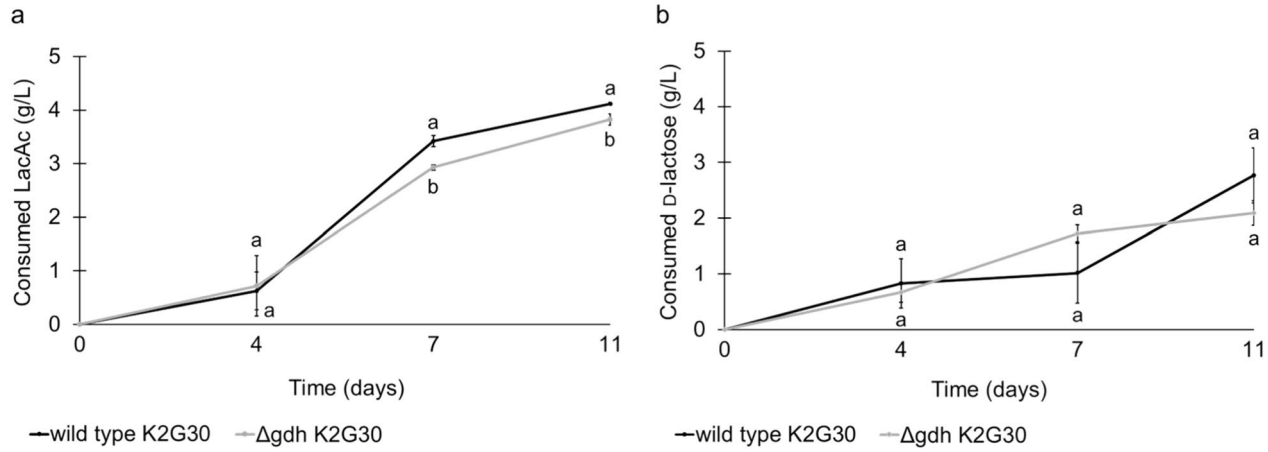

Fig. 10. D-lactic acid (LacAc) (**a**) and D-lactose (**b**) consumption rates in *wild type* and Δ*gdh* K2G30 strains on the fourth, seventh and eleventh days of growth in cheese whey (CW). Experimental data are shown as mean ± standard deviation among technical triplicates (n = 3). Different letters highlight significant differences ($p < 0.05$) between strains at the same time set point.

and enter the tricarboxylic acid cycle (TCA). In Δ*gdh* K2G30, suppressing PQQ-GDH activity led to a negligible GlcAc synthesis and a consequent inhibition of the ED pathway, limiting carbohydrate metabolism almost entirely to PP pathway fed by glucose-6-phosphate (G6P)[18]. The resulting ATP deficiency is believed to affect BC synthesis and requires an additional energy source, such as ethanol, to enhance BC production[22,23]. Once entered the cell, ethanol is first oxidized by a periplasmic alcohol dehydrogenase (PQQ-ADH) to acetaldehyde (ALD), which undergoes a subsequential oxidation to acetic acid (AcOH) by an acetaldehyde dehydrogenase[24]. AcOH feeds the TCA cycle, preserving D-glucose to be used as an energy source[25], while a quote of AcOH is released to the culture medium to modulate the environmental pH[26]. Occasionally, ethanol can be oxidized to ALD directly in the cytoplasm, thus bypassing PQQ-ADH[27]. During ethanol breakdown, AcOH accumulation causes the underexpression of ED and most of the glycolysis key genes, forcing D-glucose to be channelled in PP pathway[28]. Despite its high availability, D-glucose does not contribute to BC synthesis or biomass increase yet, because ethanol oxidation takes precedence as the primary process[25]. Once ethanol is depleted, BC synthesis and cell growth recovery occur because of a high ATP concentration[13,23], leading to an increased BC yield. Δ*gdh* K2G30, contrary to the *wild type*, seemed to be negatively affected by adding ethanol, as suggested by the lowest BC yield and D-glucose uptake shown during the first time point. According to Liu et al.[29], its low BC synthesis could be due to a failure in D-glucose transport system, since the introduction of the gene encoding for glucose facilitator protein coupled with the overexpression of the glucokinase gene led to an increased BC synthesis efficiency[29]. Furthermore, the initial D-glucose lack in Δ*gdh* K2G30 could have slowed the recovery of BC synthesis after the ethanol oxidation phase, giving the non-gluconeogenic nature of D-glucose[30]. At the end of cultivation, the mutant Δ*gdh* strain stood out for the highest BC yield and D-glucose consumption gains (Figs. 3 and 4). This behaviour is consistent with the findings of Montenegro-Silva et al.[27], which suggest that the major BC synthesis after ethanol oxidation occurs during the assimilation of AcOH[27]. Furthermore, the inactivation of PQQ-GDH did not lead to a complete termination of GlcAc synthesis (Fig. 5), presumably due to the activity of a cytosolic GDH not involved in gene silencing[31]. In *wild type* K2G30, the production of GlcAc in HS-E and HS media occurred with a similar ratio, while BC yield between media was different (Fig. 3). Since PQQ-GDH and PQQ-ADH compete for ubiquinone (UQ)[27], the concurrent oxidation of D-glucose and ethanol may have provided the *wild type* strain a higher reducing power for ATP synthesis, favouring BC production[23]. Also, the decrease in GlcAc yield shown in Fig. 5 highlighted a possible recovery of GlcAc by *wild type* K2G30 in the latest stages of growth, coherently with a deficit of energy driven by ethanol and D-glucose depletion[32].

The incapacity of Δ*gdh* K2G30 strain to benefit from ethanol addition to enhance BC synthesis required a further metabolic investigation using alternative carbon sources to D-glucose, such as D-mannitol, D-fructose, and glycerol[33–35]. The metabolism of D-mannitol occurs in the cytoplasm and starts with its oxidation to D-fructose catalysed by a heterotrimeric, soluble, mannitol dehydrogenase[36]. Next, D-fructose is further converted to F6P by phosphoenol pyruvate (PEP)-dependent fructokinase (FK)[37]. As previously described, *K. xylinus* cannot obtain fructose-1,6-bisphosphate (F16BP) from F6P due to PFK1-encoding gene deficiency, leading to a prevalent conversion of F6P to G6P by phosphoglucose isomerase (PGI)[38]. Finally, G6P can be channelled in both BC and PP pathways. Once phosphorylated to F6P, D-fructose shares the same metabolic pathway as D-mannitol[39]. Differently, glycerol undergoes a preliminary phosphorylation to glycerol-3-phosphate by a glycerol kinase (GLYK), followed by its oxidation to GA3P or dihydroxyacetone[40]; after its conversion to dihydroxyacetone phosphate (DHAP), the product can be reversely isomerized to GA3P by a phosphotriose isomerase enzyme. Following aldol condensation, both GA3P and DHAP are directed towards gluconeogenesis, where they contribute to the formation of F16BP[38]. In our study, according to Zhong et al.[39], *wild type* K2G30 strain displayed a higher D-glucose consumption than Δ*gdh* K2G30 (Fig. 7), although its BC yield at the end of cultivation was lower (Fig. 6). Furthermore, the amount of GlcAc produced by *wild type* K2G30 in HS on the seventh day was far higher than the one observed for Δ*gdh* K2G30 in the same conditions (Fig. 8), thus questioning the positive effect of GlcAc on BC synthesis reported in literature[41]. In HS-F medium, Δ*gdh* K2G30 showed a higher D-fructose uptake than the *wild type* strain at every time point (Fig. 7), even if HS achieved an overall higher BC yield. This adaptive response can be explained by considering D-fructose pathways. The energy required for PGI enzyme synthesis is believed to negatively affect BC production, thus promoting a substrate inhibition by D-fructose intermediates[42]. Furthermore, D-glucose seems to promote BC synthesis better than D-fructose through a significant stimulation of cell growth, which is positively correlated with BC yield[16]. Unlike D-glucose, D-fructose leads to a reduced influx of G6P in the BC pathway, primarily because of a decline in phosphoglucomutase activity[43]. The limited glucose-1-phosphate availability could be further responsible for the lower BC yield shown by both strains grown in HS-F. Since the phosphotransferase system is involved in D-fructose uptake, G6P generated from D-fructose through gluconeogenesis could be preferably channelled into PP pathway to replenish PEP[39], thereby reducing its availability for BC synthesis. Moreover, Δ*gdh* K2G30 strain seemed to benefit from the reduced GlcAc synthesis to support D-fructose uptake, as FK is strongly inhibited by low GA6P concentrations[42]. In previous studies, *K. xylinus* showed high BC yields when grown in glycerol and D-mannitol due to low GlcAc synthesis[15,34,35]. However, we detected only a negligible BC yield in HS-G (Fig. 6a). A similar phenomenon was previously described by Weinhouse and Benziman[44] by transferring *K. xylinus* cultures from a substrate containing D-glucose to a glycerol-rich medium. After a long adaptation phase, the organism grew fast and produced BC[44]. It was hypothesized that the inhibition of GLYK by the intracellular accumulation of F16DP led to an early delay in glycerol utilization[44]. In this study, the mutant strain showed a higher BC synthesis in HS-G after seven days of cultivation (Fig. 6b). However, this behaviour may not necessarily lead to a variation of BC productivity in the long term, which was not assessed in this work, while highlighting a different phenotypic response of the two strains. As an attempt to describe the metabolic effects of substrate switching, basing on the findings of Weinhouse and Benziman[44], we hypotesize that during the scaling-up period, the previously discussed slow uptake of D-glucose in Δ*gdh* K2G30 may have caused only marginal inhibition of GLYK by F16DP, in contrast to the *wild type*. When grown in HS-M medium, both strains displayed a variable BC yield (Fig. 6).

As reported by Anguluri et al.[37], the repeated growth of *K. xylinus* in a D-mannitol-containing medium can trigger metabolic reprogramming processes preceded by low biomass synthesis and substrate uptake, coherently with Δ*gdh* K2G30 behaviour. However, during the incubation period, the mutant strain showed a rapid increase in BC yield and D-mannitol uptake (Figs. 6 and 7). Since GlcAc synthesis in *K. xylinus* is finely regulated at gene expression level[40], we believe that *gdh* gene silencing may have induced a longer lag phase once Δ*gdh* K2G30 was transferred from HS to HS-M media, thus slowing D-mannitol uptake. CW, which identifies the main byproduct of the dairy industry resulting from the precipitation and removal of milk caseins during cheese-making[45], was tested as a substrate candidate for BC synthesis. Although numerous technologies have been developed to improve the utilization of this raw material, CW remains a significant environmental concern due to its high discarded volumes and organic matter content, while acting as a rich reservoir of various bioavailable sugars, amino acids and proteins[46] capable of promoting cellular growth and BC synthesis. Despite previous studies indicating that disaccharides are not suitable for BC production[47], using D-lactose as the primary carbon source positively impacted BC synthesis in *K. xylinus*[48]. Bacterial D-lactose metabolism requires the activity of a β-galactosidase to hydrolyse the disaccharide to D-glucose and D-galactose, which can be exploited for bothenergy production and biosynthesis[49]. Since *K. xylinus* lacks such enzyme[50], various strategies have been developed to improve D-lactose catabolic efficiency, such as substrate pretreatments[7] and the use of β-galactosidase recombinant strains[51]. In our study, a significant D-lactose uptake was detected in both Δ*gdh* and *wild type* K2G30 strains on raw CW (Fig. 10b). Assuming the absence of specific β-galattosyl-transferases, a possible D-lactose metabolic pathway may involve a first internalization through nonspecific permeases or group translocation systems, followed by its cytosolic hydrolysis[51]. According to Tonouchi et al.[52], the genome region of *K. xylinus* downstream *bcs* operon contains an 81 kDa sequence encoding a β-glycosidase, which catalyses low specificity hydrolysis reactions on various disaccharides, including D-lactose[53]. We believe that β-glycosidase activity was mainly responsible for D-lactose involvement in BC synthesis, although, to the best of our knowledge, no further evidence has been provided in literature. Contrary to D-lactose, neither strain showed a significant D-galactose consumption during incubation, as detected by HPLC analysis. According to Mikkelsen et al.[35], D-galactose is not considered a suitable carbon source for BC synthesis, due to its inefficient internalization through the cell's membrane. LacAc can stimulate cell growth and carbohydrates afflux into TCA cycle, similarly to ethanol, thus providing additional energy in the earliest growth stages[54,55]. Once entered the cell through specific permeases, LacAc undergoes an oxidation to PYR catalysed by a NAD-dependent LacAc dehydrogenase, paired with indirect ATP synthesis[56]. Since PYR does not act as a gluconeogenic substrate in *K. xylinus*[57], it is widely thought that the main influence of LacAc on BC synthesis comes from the booster effect exerted on the catabolism of other carbon sources, rather than providing intermediates available for BC synthesis[54]. As shown in Fig. 10, LacAc consumption between strains co-occurred with D-lactose uptake, although the latter seemed less preferred in the latest growth stages. In addition, only LacAc uptake highlighted significant differences between strains (Fig. 10a). Due to its relevant consumption levels, it was hypothesized an additional role of LacAc as a precursor of carbon intermediates able to feed BC synthesis. However, the specific pathways involved in this process are still unidentified. Contrary to the *wild type* strain, Δ*gdh* K2G30 displayed similar LacAc and D-lactose consumption rates during the whole incubation period, and it did not seem to suffer the initial uptake delay described for D-glucose in HS-E. Although ethanol and LacAc may stimulate energy metabolism in a similar way[55], LacAc oxidation does not lead to the synthesis of toxic intermediates like ALD, thus avoiding possible growth inhibitions[56]. Furthermore, LacAc metabolism does not require any preparation phase capable of suppressing the metabolism of other carbon sources, as confirmed by the instant uptake performed by strains (Fig. 10a). This finding suggests that LacAc may have overcome the previously reported ATP deficiency affecting Δ*gdh* K2G30 in the earliest stages of growth, leading to an anticipated BC synthesis compared to HS medium. Since the total amount of GlcAc and BC produced by strains in CW exceeds the combined amount of LacAc and D-lactose uptaken, we believe that other constituents of CW could contribute to BC synthesis. According to Yunoki et al.[25], amino acids and peptides in the culture medium can stimulate BC polymerization by providing useful D-glucose residues through gluconeogenesis. As reported by Montenegro-Silva et al.[27], gluconeogenesis could be the only process that provides D-glucose to support BC synthesis during ethanol metabolism in a *K. sucrofermentans* PQQ-GHD deficient strain. Although in this study the consumption of amino acids and peptides in CW was not assessed, previous findings suggest a possible involvement of gluconeogenesis in BC synthesis. The results obtained by growing *wild type* and Δ*gdh* K2G30 strains in raw CW could help better understand interactions between acetic acid bacteria and other microbial groups, such as lactic acid bacteria, whose exploitation in co-culture systems with *Komagataeibacter* sp. resulted in positive BC outcomes[7,58,59]. This study provided an exclusive in-depth analysis of the metabolic and phenotypic properties of *wild type* and Δ*gdh* K2G30 strains under various culture conditions, which drive their exploitation for different BC production approaches.

Methods

Used microorganisms, media, and culture conditions

Wild type K2G30 *Komagataeibacter xylinus* strain was originally isolated from Kombucha tea and deposited at Unimore Microbial Culture Collection as UMCC 2756. Its genome is available at GeneBank under the accession number QQBI00000000[34]. Before undergoing experiments, a 50% v/v glycerol cryopreserved subculture of *wild type* K2G30 has been revitalized for seven days in Hestrin-Schramm (HS) medium, composed of D-glucose 2% w/v, yeast extract 1% w/v, polypeptone 0.5% w/v, disodium phosphate anhydrous 0.27% w/v, citric acid monohydrate 0.115% w/v[60]. Solid HS-A medium was prepared by adding bacteriological agar 1.5% w/v to HS. HS-M, HS-G, and HS-F liquid media were obtained by replacing D-glucose in HS formulation with D-mannitol 2% w/v, glycerol 1.59% v/v, and D-fructose 2% w/v, respectively. HS-A-Amp and HS-Amp media were prepared with the addition of a water solution containing ampicillin 100 μg/mL to HS-A and HS formulations, respectively. HS-C

medium was prepared by adding a cellulase 1 mg/mL solution to HS medium. HS-E medium was obtained by adding absolute ethanol 1% v/v to liquid HS. CW was provided by a local cheese factory and stored at -20 °C in plastic containers before undergoing physical treatments. LB-Kan and LB-Amp media were prepared by adding 50 µg/mL kanamycin and 100 µg/mL ampicillin water solutions to Luria–Bertani (LB) medium[61], respectively. All media and materials were sterilized at 121 °C (1 atm) for 15 min before use. Ethanol, antibiotic, and cellulase solutions were microfiltered with cellulose acetate 0.22 µm filters before being added to the respective media. Experiments were carried out at 28 °C using a static cultivation system.

Construction of the vector pEASY-T5 gdh_AMPr for homologous recombination

Wild type K2G30 was first grown in HS-C medium with cellulase from *Trichoderma reesei* (Sigma-Aldrich, MO, USA) and incubated in shaking conditions until OD_{600} 0.3. Next, 5 mL of culture broth were centrifuged at 12,000 g, 4 °C, for ten minutes, and the resulting pellet was used for the DNA extraction procedure described by Gullo et al.[62]. The *gdh* gene sequence was retrieved from K2G30's genome project (BioProject accession number PRJNA482510) using scaffold annotations. Locus DV027_RS05880 on scaffold NZ_QQBI01000006.1 was selected because the putative encoded protein (Genebank accession number WP_130730967) shared 91% aminoacidic identity with the gene product of a functionally validated *gdh* gene (ATU72565) in *K. xylinus* strain CGMCC 2955[29]. Two primers, gdh-F1 and gdh-R1 (Table S6), were used to amplify a gene fragment, which was cloned into the pEASY-T5 Zero plasmid vector (TransGen Biotech Co. LTD, China) according to the suppliers' protocol. Insertion of the PCR product into the vector cloning site provided the plasmid with a unique HindIII restriction site and disrupted the encoding region of a bacterial suicide gene, facilitating identification of transformed bacterial cells. Chemically competent Trans1-T1 cells were transformed with the pEASY-T5 gdh vector by heat shock and grown on solid LB-Kan, at 37 °C overnight. Single colonies were inoculated in 5 mL of liquid LB-Kan at 37 °C for six hours and used as cell source for the vector purification (GeneJET Plasmid Miniprep Kit, Thermo Fisher Scientific, Waltham, MA, USA). The purified pEASY-T5 gdh vector was digested with HindIII and dephosphorylated with to prevent self-circularisation (FastDigest HindIII and FastAP Thermosensitive Alkaline Phosphatase, Thermo Fisher Scientific, Waltham, MA, USA). The ampicillin resistance gene amp_r was amplified with its native promoter from the plasmid pGEM®-T Easy (Promega, Madison, WI, USA), using primers that included, at their 5' ends, a recognition site for the restriction enzyme HindIII (HindIII—AMPr-F3, HindIII—AMPr-R3; Table S6). The PCR product was digested with HindIII and ligated to the pEASY-T5 gdh vector previously cleaved with the same restriction enzyme (T4 DNA Ligase, Thermo Fisher Scientific, Waltham, MA, USA). The final vector pEASY-T5 gdh_AMPr (Fig. S1a), harbouring the amp_r gene flanked by *gdh* gene sequences, was multiplied in transformed *Escherichia coli* DH 5α cells on selective LB-Amp plates and then purified from single colony inocula (GeneJET Plasmid Miniprep Kit, Thermo Fisher Scientific, Waltham, MA, USA), before sequencing at the service (Bio-Fab Research, Italy).

gdh gene silencing in K2G30

K2G30 competent cells were prepared as described by Edwards et al.[63]. 500 ng of the pEASY-T5 gdh_AMPr vector was added to a 100 µL aliquot of competent cells (~ 109 cells/mL) in a cold microcentrifuge tube. After mixing, the DNA/cell suspension was transferred to a cold 2 mm electroporation cuvette and pulsed with 2.5 kV (preset P2 program) in the Eporator gene pulser apparatus (Eppendorf, SE, Germany). Pulsed cells were supplemented with 800 µL of HS broth and transferred to a 2 mL microcentrifuge tube, before being incubated with shaking at 30 °C for 16 h. After cell recovery, 200 µL of cell culture was diluted and spread on solid HS-A-Amp containing cellulase from *Trichoderma viride* 1 mg/mL (Sigma-Aldrich, MO, USA), and incubated at 30 °C for five days. Therefore, twelve transformed K2G30 colonies have been collected and grown in 5 mL of liquid HS-Amp, at 30 °C for seven days, static. The liquid fraction of the static cell culture was centrifuged and washed with sterilized water, and the cell pellet was used for DNA extraction as described above. Disruption of the *gdh* open read frame by amp_r gene insertion was assessed by amplifying DNA regions encompassing the putative homologous recombination site (gdh-F2/gdh-R2) and the 5'/3' junctions between *gdh* target sequence and amp_r cassette (gdh-F2/AMPr-R2, gdh-R2/AMPr-F2; Table S6). The amplicon gdh-R2/AMPr-F2 was also sequenced (Bio-Fab Research, Italy). The resulting Δ*gdh* K2G30 mutant strain was deposited at UMCC under the code UMCC 3007 and cryopreserved in glycerol stocks. To check the genetic stability of the Δ*gdh* K2G30, a revitalized culture was grown on agar plates HS-A-Amp along with *wild type* K2G30 before performing the growth trials.

Cultures scaling-up and characterizations

To obtain enough culture volume for the following experiments, both Δ*gdh* and *wild type* K2G30 cultures were inoculated at 2% v/v in 50 mL HS medium contained in 100 mL Erlenmeyer flasks and incubated until $OD_{600} = 0.3$. At the end of the incubation period, both cultures underwent a morphological characterization through an optical microscope (ECLIPSE Ci-L, Nikon Corporation, Tokyo), followed by gram-staining. Biofilm pellicles were removed from the liquid phase to undergo a qualitative BC assessment, as described by Navarro and Komagata[11].

Bacterial growth in HS and HS-E media

A 2% v/v inoculum of each scaled-up culture has been performed in 50 mL HS and HS-E media contained in 100 mL Erlenmeyer flasks, respectively. The quantification of biofilm and GlcAc yields was carried out on every experimental condition on the sixth and eleventh days of incubation, along with ethanol and D-glucose consumption.

Bacterial growth in HS alternative media

Both Δ*gdh* and *wild type* K2G30 scaled-up cultures were inoculated at 2% v/v in 50 mL of HS, HS-M, HS-F and HS-G media contained in 100 mL Erlenmeyer flasks. On the fourth and seventh days of incubation, the biofilm produced in every growth medium has been collected and quantified, in parallel to GlcAc yield and each carbon source residual concentration.

Bacterial growth in cheese whey

CW was thawed at 25 °C just before use. To remove the lipidic fraction, liquid CW underwent centrifugation at 6000 g for 15 min, and the supernatant was filtered with Filter-Lab 1300/80 paper filters (Filtros Anoia S. A., Barcelona) before sterilization. A 2% v/v inoculum of each Δ*gdh* and *wild type* K2G30 scaled-up culture was performed in 50 mL sterile CW contained in 100 mL Erlenmeyer flasks and incubated for eleven days. The biofilm produced by each strain was collected and quantified on the fourth, seventh and eleventh days of growth. Analytical determination of D-galactose, D-lactose, LacAc, and GlcAc residual concentrations was also assessed.

Biofilm processing and liquid fraction purification

Biofilm pellicles produced during growth trials in CW and HS media were collected at each set time point and washed two or more times with deionized water, before being treated with NaOH 1 M at 90 °C for 30 minutes[64]. The purified biofilm was therefore washed with deionized water until reaching neutral pH. Next, pellicles were dried at 20 °C until constant weight and weighed using an E42S electronic analytical balance (Gibertini, Italy). The liquid culture separated from biofilm was filtered with PTFE 0.45 µm membranes (INCOFAR S.r.l., Italy) and diluted 1:10 with deionized water in preparation for analytical determinations.

Analytical determinations by high-pressure liquid chromatography

High-pressure liquid chromatography (HPLC) was carried out on both raw CW and purified cultures of *wild type* and Δ*gdh* K2G30 strains for every growth condition. 20 µL of the purified sample was injected in a Jasco LC-Net II/ADC HPLC apparatus (Jasco Inc., Japan) equipped with a Jasco PU-2080 Plus pump. The isocratic elution was carried out using an Aminex® HPX-87H column (Bio-Rad Laboratories, Italy) heated at 40 °C with an Eldex CH-150 oven (Eldex Corp., USA). The mobile phase (H_2SO_4 0.005 N + acetonitrile 5% v/v) was flown through the column at a 0.6 mL/min flux rate[7]. The detection of organic acids and carbohydrates was performed using a Jasco UV-2070 Plus UV detector and a Jasco RI-2031 Plus IR detector, respectively.

Data analysis

The statistical data analysis was performed through one-way analysis of variance (ANOVA) using the multcompView packet implemented in R v. 4.2.2[65]. The differences between means were evaluated through Tuckey HSD test ($p < 0.05$). If not specified, every trial was executed as a technical triplicate.

Data availability

All data generated or analysed during this study are included in this published article and its supplementary information files.

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Author contributions

M.G. and S.C. conceived the idea; F.L. and S.C. designed the experiments; F.L. and S.C. conducted the experiments; F.L. wrote the first version of the manuscript; M.G. and S.C. assisted in writing the manuscript; M.G. provided funding. All authors contributed to the revision of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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