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Influence of dietary disaccharides on glycosyl hydrolase profile in ecologically distinct Lactobacillus strains

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Abstract. This study examines glucosyl hydrolase enzymes from Lactobacilli strains isolated from probiotic foods and beverages (kefir, pickles and sauerkraut). Glycosyl hydrolases play a key role in the breakdown of complex carbohydrates, supporting gut health and finding wide biotechnological applications. During the screening stage, the metabolic potential of the strains to utilize various carbohydrates in modified, glucose-free media containing specific carbon substrates is tested. This points to the search for specific enzymes such as α-/β-glucosidase and α-/β-galactosidase. The results indicate that glycosidase activity in *Lactobacillus* varies by strain and is influenced by the available substrate, highlighting their potential applications in functional foods, prebiotic conversion, and the metabolism of polyphenolic compounds.

Key words: glycosyl hydrolases, *Lactobacillus*, lactulose, cellobiose, lactose.

Introduction

The genus *Lactobacillus* comprises a diverse group of Gram-positive, facultatively anaerobic bacteria that are widely recognized for their role in food fermentation, host health, and ecological adaptability (Axelsson, 2004; Cai et al., 2009). These bacteria are prominent members of the microbiota in a variety of habitats, including the gastrointestinal tract, the oral cavity, the vaginal mucosa, and fermented foods (Walter, 2008; Zheng et al., 2020). Their ecological success and functional versatility are largely attributed to their extensive metabolic capabilities, including a broad arsenal of enzymes that allow them to interact with complex substrates in their environment (Maske et al., 2021).

Among these enzymatic functions, glycosyl hydrolases and related enzymes involved in carbohydrate and different types of glycosides metabolism are of particular interest. These enzymes not only enable *Lactobacillus* strains to utilize diverse dietary components but also contribute to host health by modulating bioavailability and activity of bioactive compounds such as polyphenols (Esteban-Torres et al., 2013; Selma et al., 2009). Notably, enzyme expression and activity in *Lactobacillus* are highly niche-dependent, reflecting adaptation to specific environmental pressures and available substrates (Martino et al., 2016). As such, strains isolated from fermented plant-based foods, breast milk, or mucosal surfaces may display unique enzymatic profiles aligned with their ecological origin.

Fermented and functional foods often serve as a natural source of *Lactobacillus* species, which are commonly used as probiotics due to their

health-promoting properties (de Melo Pereira et al., 2018). Functional foods provide scientifically substantiated health benefits beyond basic nutrition (Hasler & Brown, 2009). These benefits can include disease prevention or health promotion, delivered through added or inherent bioactive compounds or live microorganisms at effective and safe levels (Frumuzachi et al., 2025). Fermented foods serve as a rich ecological niche for *Lactobacillus* species due to their unique biochemical environment. They contain carbohydrates (e.g., lactose, glucose, fructose, etc.) that lactobacilli readily ferment to produce lactic acid, acetic acid and other organic acids. The acidification caused by lactic acid favors acid-tolerant bacteria like *Lactobacillus*, while inhibiting many pathogens (Praveen & Brogi, 2025). Over time, lactobacilli evolve strain-specific adaptations to particular substrates (e.g., milk proteins, plant fibers) and develop functional roles such as exopolysaccharide production, polyphenol metabolism and vitamin biosynthesis (Crittenden et al., 2003; Marco et al., 2017).

Disaccharides such as lactose, lactulose, and cellobiose play significant roles in shaping microbial carbohydrate metabolism, particularly in *Lactobacillus* spp. Lactose, a naturally occurring β-1,4-linked disaccharide of glucose and galac-tose, is abundant in milk and a primary carbon source for many intestinal microbes (Xu et al., 2024). Lactulose, a synthetic isomer of lactose with a β-1,4 linkage between galactose and fructose, is not digested by human enzymes but selectively stimulates the growth of beneficial gut bacteria, including *Lactobacillus* (Rasoulimehrabani et al., 2025; Wang et al., 2024). Cellobiose, a β-1,4-linked glucose disaccharide derived from cellulose degradation, is less common in the human diet but serves as a model substrate for β-glucosidase activity in microbial systems (Van Zanten et al., 2015). These structurally related disaccharides can act as inducers of specific glycosyl hydrolases, influencing enzymatic expression profiles and metabolic adaptation in *Lactobacillus* strains from diverse ecological niches.

Understanding niche-specific enzyme sets is essential for elucidating the ecological roles and functional potential of *Lactobacillus* strains, particularly in the context of developing next-generation probiotics or tailored fermentation processes (Kumari et al., 2021). Despite growing interest in the functional genomics of *Lactobacillus*, detailed biochemical characterizations of enzyme activity across ecologically diverse strains remain limited.

In this study, we investigate the enzyme profiles of *Lactobacillus* strains isolated from fermented foods and beverages. We characterized glycolsyl hydrolase activities, as a part of their probiotic potential in the presence of different carbohydrate sources such as cellobiose, lactose, and lactulose, to explore correlations between ecological origin and enzymatic capacity.

Materials and methods

Bacterial strains

The *Lactobacillus* strains used in this study were isolated from traditional fermented foods and beverages, including kefir, pickles, and sauerkraut. From over 20 initial isolates, a subset of representative strains was selected based on preliminary screening. Biochemical and microbiological identification confirmed that all selected strains belong to the genus *Lactobacillus.*

Identification of Lactobacillus strains

After initial culturing in MRS broth (De Man et al., 1960; VWR, USA) at 30°C under anaerobic conditions for 24 hours, colonies with distinct morphologies were selected for further identification. Gram staining was performed to verify that the isolates were Gram-positive rods, and catalase activity was assessed by applying 3% hydrogen peroxide to fresh cultures; all isolates tested were catalase-negative, consistent with *Lactobacillus* spp. For molecular identification, genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), and the 16S rRNA gene was amplified using primers: 8F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 15R (5′-AAGGAGGTGATCCARCCGCA-3′) (Eurogentec, Belgium). PCR products were purified and sequenced, and the resulting sequences were compared against the NCBI GenBank database using BLAST.

Growth medias

The medium used for cultivating the LAB strains was MRS broth, selective for *Lactobacillus* (VWR). For carbohydrate utilization analysis, a modified MRS broth was prepared by omitting glucose and supplementing it with the tested substrates as it follows: 4% lactose or lactulose; and 1% FOS, cellobiose or inulin.

Carbohydrate utilization profiles

The ability of the selected strains to utilize the carbohydrates was assessed as follows: modified MRS media containing the respective carbohydrate sources were inoculated with 10% (v/v) of an overnight culture grown in standard MRS broth. Cultures were incubated at 30 °C under anaerobic conditions for 24 hours. Standard MRS medium without carbohydrate modification served as the control. After incubation, pH was measured to assess acid production, and viable cell counts were determined by plating serial dilutions on standard MRS agar to evaluate bacterial growth.

Enzymatic assays

Enzymatic activities of the strains were assessed following cell lysis. After cultivation in modified MRS broth containing either 4% lactulose, 4% lactose, or 1% cellobiose, cells were harvested by centrifugation and resuspended in 1 mL of disintegration buffer (0.02 M sodium acetate buffer, pH 6.0, containing 0.1 M NaCl and 2% glycerol). Cultures were incubated for either 6 or 16 hours to compare the enzymatic activities at different growth stages. Cell lysis was performed using an UP50H Ultrasonic Processor (Hielscher Ultrasound Technology, Germany). The lysates were then centrifuged, and the resulting supernatants were collected for enzymatic assays. The activities of α-galactosidase, β-galactosidase, α-glucosidase, and/or β-glucosidase were measured using spectrophotometric methods described bellow. All enzyme activity experiments were performed in triplicate.

The α-galactosidase activity was measured using the method described by Petek et al. (1969), with slight modifications. The assay involved quantifying the release of p-nitrophenol (pNP) from the degradation of pNP-α-D-galactopyranoside (Sigma-Aldrich). The reaction mixture consisted of 250 µL of 2 mM pNP-α-D-galactopyranoside substrate in 100 mM sodium acetate buffer (pH 6.0) and 100 µL of bacterial lysate. The final volume was adjusted to 500 µL with distilled water, and the mixture was incubated for 20 minutes at 30°C. The reaction was terminated by adding 2 mL of 1 M Na_2CO_3. The released pNP was then measured spectrophotometrically at 405 nm.

The β-galactosidase activity was measured using the method of Lim & Chae (1989), with slight modifications. The assay involved quantifying the release of o-nitrophenol (oNP) from the degradation of oNP-β-D-galactopyranoside (Sigma-Aldrich). The reaction mixture consisted of 250 µL of 2 mM oNP-β-D-galactopyranoside substrate in 100 mM sodium acetate buffer (pH 6.0), 100 µL of bacterial lysate, and 150 µL of distilled water. The mixture was incubated for 20 minutes at 30°C. The reaction was terminated by adding 2 mL of 1 M Na_2CO_3. The released oNP was then measured spectrophotometrically at 405 nm.

The α-glucosidase activity was measured using the method of Dewi et al. (2007), with some modifications. The assay involved quantifying the release of p-nitrophenol (pNP) from the degradation of pNP-α-D-glucopyranoside (Sigma-Aldrich). The reaction mixture included 250 µL of 1.25 mM pNP-α-D-glucopyranoside substrate in 100 mM sodium acetate buffer (pH 6.0) and 100 µL of bacterial lysate. The final volume was adjusted to 500 µL with distilled water, and the mixture was incubated for 20 minutes at 30°C. The reaction was terminated by adding 2 mL of 1 M Na_2CO_3. The released pNP was then measured spectrophotometrically at 405 nm.

The β-glucosidase activity was measured using the method of Martin & Akin (1988), with some modifications. The assay involved quantifying the release of p-nitrophenol (pNP) from the degradation of pNP-β-D-glucopyranoside (Sigma-Aldrich). The reaction mixture consisted of 250 µL of 20 mM pNP-β-D-glucopyranoside substrate in 100 mM sodium acetate buffer (pH 5.0) and 100 µL of bacterial lysate. The final volume was adjusted to 500 µL with distilled water, and the mixture was incubated for 10 minutes at 30°C. The reaction was terminated by adding 2 mL of 1 M Na_2CO_3. The released pNP was then measured spectrophotometrically at 405 nm.

Protein content

The protein content in all samples was measured using the Bradford method (1976), with bovine serum albumin (Sigma-Aldrich) as a standard. Spectrophotometric measurements were conducted using a Shimadzu UV-2600 spectrophotometer (Shimadzu Corp., Kyoto, Japan), and all experiments were carried out in triplicate.

Results

Identification of Lactobacillus strains

16S rRNA gene sequencing was performed using the 8F primer, and the resulting sequences

were compared against the NCBI GenBank database using BLAST. The results showed 94–97% identity with *Lactobacillus plantarum*, except for strain Z5, which matched *Lactobacillus pentosus* (Table 1).

Table 1. Identification of isolates obtained from functional foods.

Strain ID	Species	Similarity	Origin
L.pl.KG38	*Lb. plantarum*	94.62%	Kefir
L.pl. KG62	*Lb. plantarum*	97.05%	Kefir
L.pl. K2	*Lb. plantarum*	95.44%	Pickles
L.pl. K7	*Lb. plantarum*	96.71%	Pickles
L.pent. Z5	*Lb. pentosus*	95.51%	Sauerkraut
L.pl. K8	*Lb. plantarum*	95.66%	Pickles

Carbohydrate utilization profiles

To evaluate the ability of the selected *Lactobacillus* strains to utilize various carbohydrates, their growth and the acidification of the media were assessed after 24 hours of incubation in modified MRS media containing 1% fructooligosaccharides (FOS), 1% inulin, 2% cellobiose, 4% lactose, or 4% lactulose. All strains showed robust growth in the standard MRS medium (Table 2). Among the tested carbohydrates, lactose and lactulose supported the highest cell densities in most strains, particularly KG38, KG62, and K8, with final counts exceeding 3.8×10^9 CFU/mL. In contrast, inulin and FOS resulted in lower growth overall, with inulin supporting the least growth (as low as 2.0×10^8 CFU/mL in K8), and moderate acidification (pH values between 5.61 and 5.83).

These results demonstrate that lactose, lactulose, and, in some cases, cellobiose serve as efficient fermentable substrates for the tested strains, while FOS and inulin support limited growth and acid production under the given conditions.

Table 2. Cell growth measured as CFU/mL in standard and carbohydrate-supplemented MRS broth.

Strain	MRS	1% FOS	1% Inulin	2% Cellobiose	4% Lactose	4% Lactulose
L.pl. KG38	1.18×10^{10}	1.8×10^9	4.0×10^8	5.1×10^9	6.45×10^9	9.1×10^9
L.pl. KG62	1.095×10^{10}	2.25×10^9	6.5×10^8	1.43×10^{10}	6.25×10^9	3.85×10^9
L.pl. K2	2.7×10^9	3.0×10^8	2.5×10^8	1.2×10^9	2.0×10^8	1.0×10^8
L.pl. K7	4.75×10^9	3.55×10^9	4.0×10^8	4.0×10^8	5.0×10^7	2.0×10^8
L.pent. Z5	5.45×10^9	1.8×10^9	4.5×10^8	7.0×10^8	2.0×10^8	2.5×10^8
L.pl. K8	4.05×10^9	8.5×10^8	2.0×10^8	2.3×10^9	6.5×10^8	7.5×10^8

Enzyme profile of the six studied Lactobacillus strains

Enzyme profile in lactulose-based MRS media

Enzyme profiling of lactobacilli in the presence of lactulose provides valuable insights into their carbohydrate metabolism and potential probiotic functions. Since lactulose is a synthetic disaccharide composed of galactose and fructose, its utilization requires specific enzymatic activities, such as β-galactosidase, which hydrolyzes lactulose into its monosaccharide components. In this study, the substrate served as a starting point, providing insights into which enzyme activities were most prominent and guiding the selection of subsequent assays. This approach helps in understanding strain-specific differences in enzyme secretion, particularly those related to prebiotic utilization and gut health benefits.

Cell-associated enzymatic activity of α-galactosidase, β-galactosidase, α-glucosidase, and β-glucosidase were spectrophotometrically measured. Enzyme secretion was compared between the early and late exponential phases under same conditions: 4% lactulose, 30°C, for all studied strains.

The comparative bar chart clearly illustrates distinct enzyme activity profiles among the tested strains over time. β-Galactosidase exhibited the highest activity overall, with all strains showing a notable increase between 6 and 16 hours. Strains K7, K8, Z5, and K2 reached activities above 0.25 U/mg at 16 hours, indicating strong induction during prolonged incubation. α-Galactosidase activity also tended to rise between 6 and 16 hours in most strains, especially in KG62, which had the highest recorded activity (0.176 U/mg protein), suggesting enhanced metabolism of α-galactoside substrates in this strain. In contrast, α-glucosidase activity remained consistently low in all strains which may imply limited involvement of this enzyme under the tested conditions. β-Glucosidase activity showed more variability, with reduced activity among most of the strains at 16h, indicating potential differences in enzyme regulation (Fig. 1).

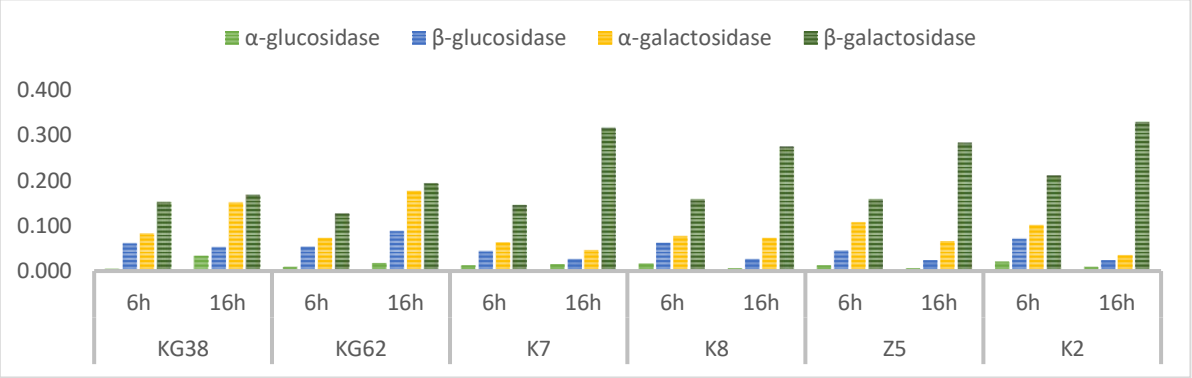

Fig. 1. Specific enzymatic activity of six studied *Lactobacillus* strains, cultivated in modified MRS media containing 4% lactulose, measured at 6 and 16 hours.

Enzyme profile in cellobiose-based MRS media

To assess the inducing capacity of cellobiose on beta-glucosidase activity, all the strains were cultivated in modified MRS broth containing 1% cellobiose, and incubated at 30°C for 6 or 16 hours. Results were compared to the previously obtained when lactobacilli were grown on lactulose (Fig. 2).

Without a doubt, cellobiose is a more effective inducer of β-glucosidase than lactulose. β-Glucosidase activity varied among the six *Lactobacillus* strains, indicating strain-dependent differences in β-glucosidase regulation in response to cellobiose. While strains K2, K7, and K8 showed increased activity from 6 to 16 hours—suggesting possible induction or sustained enzyme expression—KG38 and KG62 exhibited a marked decrease over time. Strain Z5 maintained relatively stable activity. These results clearly illustrate the differences in enzymatic activity related to the origin of the lactobacilli.

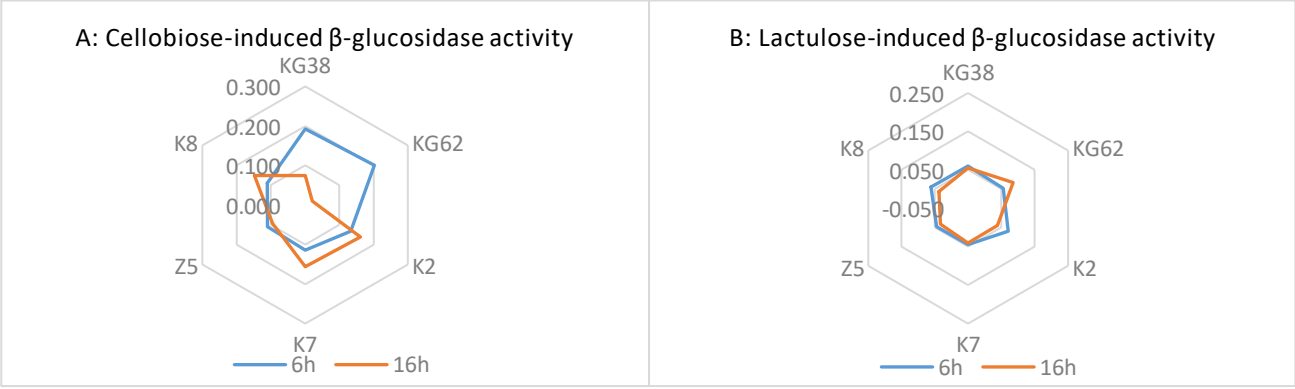

Fig. 2. Beta-glucosidase activity of six *Lactobacillus* strains isolated from fermented foods and beverages, cultivated in modified MRS media containing 1% cellobiose (A) or 4% lactulose (B), measured at 6 and 16 hours. Enzyme activities are expressed in U/mg protein.

Beta-galactosidase activity in presence of lactose vs. lactulose as a C-substrate

As a common natural disaccharide, lactose was included in the enzyme assays. The β-galactosidase activities were assessed and compared with those detected in strains previously cultivated on the synthetic disaccharide lactulose. All strains were grown in modified MRS broth containing 4% lactose and incubated at 30 °C for 6 or 16 hours. All strains demonstrated enzymatic activity at both time points, with an overall increase observed at 16 hours compared to 6 hours (Fig. 3).

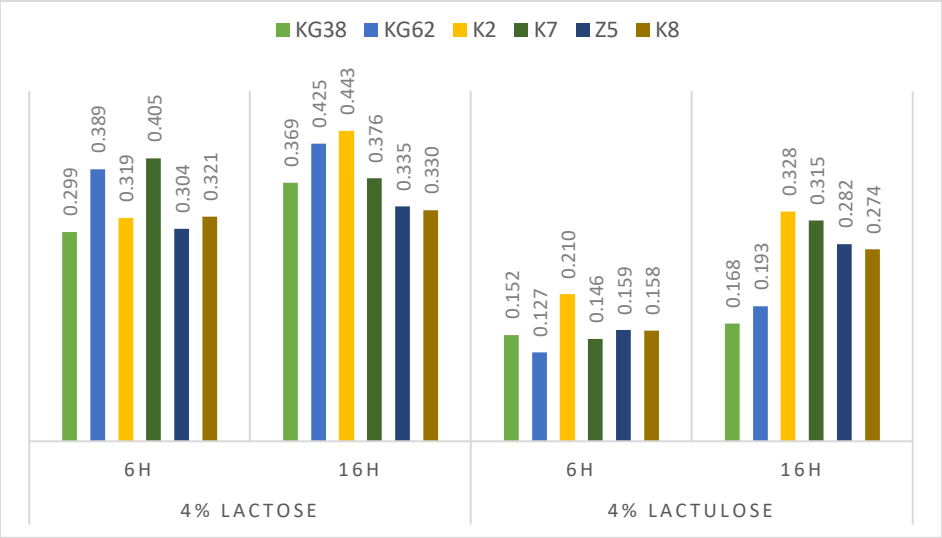

Fig. 3 Specific β-galactosidase activity of six studied *Lactobacillus* strains, cultivated in modified MRS medium containing 4% lactose or 4% lactulose. The chart illustrates differences in β-galactosidase induction by the natural disaccharide lactose versus the synthetic disaccharide lactulose.

Overall, lactose proved to be a stronger inducer of β-galactosidase than lactulose, with higher enzyme activities observed across all strains at both 6 and 16 hours. Strain K2 exhibited the highest activity on both substrates, reaching 0.443 U/mg protein with lactose and 0.328 U/mg protein with lactulose at 16 hours. Most strains—KG38, KG62, K2, Z5, and K8—showed clear increases in activity from 6 to 16 hours, while K7 was the only strain to display a slight decline, decreasing from 0.405 to 0.376 U/mg, suggesting peak enzyme expression occurred earlier. These findings underscore the strain-specific enzymatic profiles and indicate that natural disaccharides like lactose may be more effective in inducing β-galactosidase production in lactobacilli. Additionally, lactose supported earlier induction, as all strains reached activities between 0.299 and 0.405 U/mg by the 6-hour time point.

α-Galactosidase activity was also assessed in lactose-enriched medium. Strains K2, K7, K8, and Z5 showed no detectable activity at either 6 or 16 hours. In contrast, strains KG38 and KG62, both isolated from kefir, exhibited very low activity levels (0.04–0.06 U/mg) after 16 hours of cultivation. These results suggest that α-galactosidase is involved primarily in the metabolism of lactulose and is not effectively induced in the presence of lactose.

Discussion

The tested *Lactobacillus* strains displayed distinct carbohydrate utilization profiles, with lactose, lactulose, and cellobiose supporting the most robust growth and acidification, particularly in strains KG38, KG62, and K8. In contrast, limited growth on inulin and FOS suggests these prebiotics are less readily metabolized under the tested conditions.

Lactulose proved to be a suitable substrate for glycosyl hydrolase profiling. The enzymatic profiles of six *Lactobacillus* strains isolated from fermented foods and beverages revealed notable strain- and substrate-specific differences in glycolsyl hydrolase activity when cultivated on lactulose. α-Glucosidase activity remained low and relatively unchanged across strains and time points,

suggesting minimal induction under the tested conditions or limited involvement of this enzyme in lactulose metabolism. In contrast, β-galactosidase activity was consistently the highest, particularly at 16 hours, indicating progressive adaptation of the strains to the surrounding conditions.

Lactulose is often applied as a substrate for exopolysaccharide (EPS) production by lactic acid bacteria. Different LAB species can produce a wide variety of EPS with notable technological and nutritional functionality (Mozzi et al., 2009; Ruas-Madiedo et al., 2002). A study of Fara et al. from 2020 reported the successful synthesis of oligosaccharides from lactose (GOS) and lactulose (OsLu) with a potential prebiotic effect by *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL450. GOS are defined as nondigestible carbohydrates with proven modulating effect of the colonic microbiota toward a healthy balance (Macfarlane et al., 2008). In 2023 Fuso et al. have studied the effect of different sugars on EPS production and their molecular characteristics. This suggests opportunities for the future synthesis of tailored EPS (Fuso et al., 2023).

Lactose further confirmed its role as a natural and effective inducer of β-galactosidase, not only triggering higher enzyme activity but also promoting earlier induction. However, minimal to no α-galactosidase activity was detected in lactose-based media. In contrast, measurable α-galactosidase activity in lactulose-based media suggests either broader substrate specificity or possible co-regulation with other glycosidases.

A comparison of β-glucosidase activity in lactulose- versus cellobiose-supplemented media clearly demonstrated enhanced enzyme expression in the presence of cellobiose, highlighting its stronger inductive effect. Notably, niche-dependent patterns emerged: strains KG38 and KG62 (from kefir) showed a decline in activity over time, while strains K2, K7, and K8 (from pickles) exhibited a marked increase at 16 hours. Strain Z5 (from sauerkraut) maintained relatively stable activity levels throughout the cultivation.

Comparative genomics has greatly advanced our understanding of LAB evolution. Since the first genome sequence of *Lactobacillus plantarum* WCFS1 was published in 2003 (Kleerebezem et al., 2003), more than 15 *Lactobacillus* genomes, spanning over a dozen species, have been released. A comparative study of nine LAB genomes by Makarova et al., 2006 revealed that their adaptation to diverse environments involved both gene acquisition and gene loss. A study of Sun et al. (2015) puts emphasis on lactobacilli's "encoded genetic catalogue for modifying carbohydrates and proteins". Through comparative genomics of 213 *Lactobacillus* strains from various genera, they report that the 213 genomes collectively encode 48 of the 133 families of glycoside hydrolases (GHs) in the CAZy database (http://www.cazy.org), many of which represent unrecognized and unexploited enzymes for biotechnology.

Both the genetic repertoire and the regulatory mechanisms are crucial for explaining *Lactobacillus* metabolism and adaptation. A recent article of Echegaray et al. (2023) summarizes the advances in the omics approaches to clarify the roles and mechanisms underlying the functional properties of *Lactobacillus plantarum*. Omics technologies have clarified how genetic information shapes microbial phenotypes, linking genomes to cellular metabolites. Transcriptomics, proteomics, and metabolomics provide complementary insights at the mRNA, protein, and metabolite levels, respectively (Manzoni et al., 2018). Unlike traditional methods, these approaches must be integrated, as no single technique can fully capture the complexity of an organism.

Conclusions

In conclusion, the findings indicate that glycosidase activity in *Lactobacillus* varies by strain and is influenced by the available substrate, highlighting their potential applications in functional foods, prebiotic conversion, and the metabolism of polyphenolic compounds. Future studies should investigate the gene-level regulation of these enzymes, evaluate their specificity toward different substrates, and assess their functional performance in more complex food systems.

Acknowledgments

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