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High temperature lacto-fermentation improves antioxidant and antidiabetic potentials of Lithuanian red beetroot

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ABSTRACT

Diabetes is becoming a global pandemic and therefore calls for diverse therapeutic strategies for mitigating the disease. In this study, lactic acid bacteria isolated from Lithuanian traditional fermented foods were screened for β-Glucosidase activity and were used to ferment beetroot to develop an antidiabetic product. After fermentation, LAB PN39MY (also referred to as PN39) fermented samples demonstrated the strongest DPP-IV and α-Glucosidase inhibitory abilities of 50.0 ± 3.5% and 80.5 ± 5% respectively as well as the highest antioxidant capacity of 0.69 ± 0.04 mmol/L relative to all the other samples. Varying the fermentation conditions revealed that fermenting beetroot with PN39 at 45 °C for 72 h yielded the strongest DPP-IV and α-Glucosidase inhibition of 87.45% and 80.5 ± 5.3% respectively and increased the antioxidant capacity to 1.46 ± 0.01 mmol/L. Whole genome sequencing analysis of PN39 revealed the strain to be *Latilactobacillus curvatus*. HPLC-MS analysis of PN39 fermented samples showed that the fermentation process generated high levels of dihydromyricetin (an antidiabetic flavonoid) which was absent in unfermented beetroot. Results from this study indicate that fermenting beetroot with *Latilactobacillus curvatus* PN39 at 45 °C for 72 h would be an effective method for developing antidiabetic functional foods.

1. Introduction

Type 2 diabetes mellitus (T2D) is a chronic metabolic disease characterized by hyperglycemia which results in damaged pancreatic beta cells and insulin resistance (Bellary, Kyrou, Brown, & Bailey, 2021). The increasing burden of T2D is of great concern to global health care systems as about 8% of the world's population are affected by the disease (Khan et al., 2020). Prolonged exposure to high blood glucose levels has been associated with the overproduction of reactive oxygen species (ROS) which induce oxidative stress, a major player in T2D pathophysiology (Bhatti et al., 2022). In fact, hyperglycemia-induced ROS contributes to diabetes complications such as nephropathy (Chun & Park, 2020), cardiovascular events as well as micro- and macrovascular diseases (Mannucci, Monami, Lamanna, & Adalsteinsson, 2012). Also, T2D leads to an increased production of dipeptidyl peptidase-IV (DPP-IV) which inhibits the body's incretin system responsible for maintaining glucose homeostasis (Kasina & Baradhi, 2022). Glycemic control is therefore an important therapeutic strategy for T2D management. In the gut, pancreatic α-Amylase and intestinal α-Glucosidase hydrolyze dietary carbohydrate to release glucose. For this reason, retarding the release of dietary glucose by inhibiting the activities of these enzymes has become an effective therapeutic strategy for controlling postprandial hyperglycemia (DiNicolantonio, Bhutani, & O'Keefe, 2015). Yet, existing pharmacological therapeutics that inhibit carbohydrate-digesting enzymes can have many side effects including gastrointestinal discomfort, liver disorders and nausea (DiNicolantonio et al., 2015). However, the prevalence of diabetes, coupled with the side effects of existing antidiabetic drugs have necessitated the search and development of safe non-pharmacological therapeutics for the management of the disease. Meanwhile, there is increasing evidence that natural compounds such as carotenoids, anthocyanins and betalains found in vegetables such as red beetroot have potential antioxidant and

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glycemic control effects (Azizah, Emelda, Asmaliani, Ahmad, & Fawwaz, 2022; Li et al., 2022; Tian et al., 2021).

Fermented red beetroot (*Beta vulgaris* L.) is a popular part of traditional Lithuanian delicacies such as Šaltibarščiai and pickled beetroot. The vegetable is rich in betalains, which are known for their anti-inflammatory, antioxidant and antidiabetic effects in both animal (Olasehinde, Oyeleye, Ibeji, & Oboh, 2022) and human studies (Aliahmadi et al., 2021; Karimzadeh, Behouz, Sohab, Hedayati, & Emami, 2022). Most *in vitro* (Oboh, Obayiuwana, Aihie, Iyayi, & Udoh, 2020) and *in vivo* studies regarding the antidiabetic abilities of beetroot have focused on fresh beetroot (Aliahmadi et al., 2021; Karimzadeh et al., 2022; Olasehinde et al., 2022) but little is known about the antidiabetic potentials of fermented beetroot. Meanwhile, several studies have shown that fermentation with lactic acid bacteria can activate, transform and improve the availability of bioactive compounds to augment their health promoting effects (Michlmayr & Kneifel, 2014; Rodríguez et al., 2021; Zhao et al., 2021). In fact, some studies have shown that fermentation may liberate bound bioactive beetroot dyes during fermentation and this may account for the high pigment compounds in fermented beetroot relative to unfermented samples (Janiszewska-Turak et al., 2022; Czyżowska, Klewicka, & Libudzisz, 2006). Interestingly, lactic acid bacteria with active β-Glucosidase can hydrolyze β-D-glucosyl residues of betanin (the main red-violet component in beetroot) to yield aglycones such as betanidin and isobetanidin (Czyżowska et al., 2006) which demonstrate improved biological activities than their parent compound (Wybraniec et al., 2011). Most of the metabolites generated after the fermentation process are usually readily absorbed though the gut epithelium via passive diffusion (Xiao & nutrition, 2017) thereby increasing their bioavailability. For this reason, identifying specific lactic acid bacteria that can improve the antidiabetic potentials of beetroot could be a better strategy for developing functional foods with reproducible health effects relative to uncontrolled spontaneous fermentation. In this study, we isolated lactic acid bacteria from Lithuanian traditional fermented tomatoes, orange, pear and pickles for the purpose of developing a beetroot product with strong antidiabetic potentials. The bacteria were screened based on their ability to survive in beetroot juice, their β-Glucosidase activities and the ability of their fermented products to demonstrate antidiabetic potentials (α-Glucosidase, α-Amylase, DPP-IV inhibition and antioxidant activities). The bacterium whose fermented product demonstrated the strongest antidiabetic potentials was identified by whole genome sequencing and the metabolites in the sample were assessed by liquid chomatography–mass spectrometry (HPLC-MS). Results from this study would give insights into how novel strains such as *Latilactobacillus curvatus* PN39 isolated from traditional fermented foods could be useful for developing antidiabetic functional foods.

2. Materials and methods

2.1. Chemicals and reagents

Unless specified, all the media and reagents were purchased from Sigma-Aldrich Sp. z o.o. (Poznań, Poland). β-Glucosidase assay kit, α-Glucosidase, α-Amylase and DPP-IV inhibitor screening kits were purchased from Abcam (Cambridge, UK). Total antioxidant capacity assay kit was purchased from Sigma-Aldrich Sp. z o.o. (Poznań, Poland).

2.2. Materials and bacteria isolation

Red beetroot (*Beta vulgaris* L.), locally fermented tomatoes, sauerkraut and pickles were bought from Kalvariju market (Kalvarijų turgus, Vilnius- Lithuania). All the samples were placed on ice and transported to the laboratory. For lactic acid bacterial isolation, 100 μL of the liquid in each fermented sample was spread on De Man, Rogosa and Sharpe (MRS) agar and incubated aerobically at 37 °C for 48 h. Bacteria colonies were separated based on their morphological differences and single colonies were inoculated in MRS broth and incubated at 37 °C for 48 h under aerobic conditions. An aliquot (100 μL) of the overnight cultures was spread on MRS agar and incubated at 37 °C for 48 h under aerobic conditions. Pure isolates were identified by microscopic and phenotypic tests. The bacteria strains were tested for their ability to grow in the presence of beetroot juice using the agar well diffusion test. In all, 22 isolates were resistant to beetroot juice. These strains were selected and stock cultures were prepared in MRS broth containing 20% glycerol (v/v). The bacteria stocks were maintained at −80 °C.

2.3. Selection of lactic acid bacteria based on their β-Glucosidase activities

Frozen bacteria cultures were revived by streaking on MRS agar and incubated at 37 °C for 24 h. MRS broth was inoculated with single colonies of bacteria, incubated at 37 °C and harvested at the exponential phase of growth. The bacteria were screened for their β-Glucosidase activity using a β-Glucosidase assay kit according to the manufacturer's instructions. Briefly, overnight cultures of each strain was vortexed and 50 μL of culture were added to 200 μL of working reagent containing *p*-nitrophenyl-β-D-glucopyranoside. The mixture was incubated at 37 °C for 8 h and the optical density was read at 405 nm with SpectraMax i3 Multi-Mode Detection Platform (REUZEit, LLC, Temecula, CA 92590 USA). Bacteria that showed the lowest β-Glucosidase activity were eliminated from further studies.

β-Glucosidase activity of the sample (μkat) was calculated as:

$$\beta - \text{Glucosidase Activity} = \left(\frac{OD8h - OD0h}{ODcalibrator - ODH20}\right) X\ 250(\mu kat)$$

Where OD_{0h} = Optical density at 405 nm at time 0 h

OD_{8h} = Optical density at 405 nm at time 8 h
$OD_{calibrator}$ = Optical density of calibrator at 405 nm at time 8 h
OD_{H20} = Optical density of water at 405 nm at time 8 h

2.4. Preparation of beetroot and fermentation

The beetroot (150 g) was washed with distilled water to remove surface dirt and blanched at 100 °C for 5 min to inactivate surface microorganisms and enzymes. The skin was peeled and sliced and 100 mL of distilled water was added to 100 g of the sample. The sample was blended using a Philips H3664/90 blender (Vilnius, Lithuania). Fermentation was carried out as described by Czyżowska et al. (Czyżowska et al., 2006) with modifications. Briefly, each selected lactic acid bacterium (2×10^8 cfu/mL) was inoculated into beetroot pulp (200 g) as the sole energy source and incubated at 37 °C for 48 h at 150 rpm agitation. The fermented sample was centrifuged at 8000×*g* for 5 min and the supernatant was stored at −80 °C for further studies.

2.5. In vitro antidiabetic activity of fermented beetroot

2.5.1. DPP-IV inhibitory ability

DPP-IV inhibitory ability was assessed using a DPP-IV inhibitor screening kit according to the manufacturer's instructions. Briefly, 10 μL of fermented beetroot samples were mixed with 10 μL of diluted DPP-IV and transferred to wells containing 50 μl of diluted DPP-IV substrate. The plate was incubated at 37 °C for 30 min and fluorescence was measured at excitation wavelength of 350–360 nm and an emission wavelength of 450–465 nm. Sitagliptin was used as a positive control inhibitor. Samples whose inhibitory abilities were stronger than fresh beetroot (unfermented sample) were chosen for further studies.

DPP-IV inhibitory ability was calculated as:

$$\%\ \text{Inhibition} = \left(\frac{Activity\ (without\ inhibitor) - Activity\ (with\ inhibitor)}{Activity\ (without\ inhibitor)}\right) X\ 100\%$$

2.5.2. Total antioxidant capacity

Total antioxidant activity was analyzed using a Total Antioxidant Capacity Assay Kit according to the manufacturer's instructions. This kit indirectly estimates the concentration of small molecule antioxidants in the test sample that can reduce Cu^{2+} ion to Cu^{+}. The amount of reduced Cu^{+} ions generated is proportional to the total antioxidant capacity of the test sample. To determine the total antioxidant capacity of the beetroot samples, 100 μl of dilute samples were mixed with 100 μL of the Cu^{2+} working solution and incubated at room temperature (~25 °C) for 90 min. Trolox was used as the standard antioxidant and absorbance was measured at 570 nm. Samples with total antioxidant concentrations greater than fresh beetroot were chosen for further studies. Concentration of antioxidants in each sample was calculated as trolox equivalents as $S_a/S_v = C$

Where S_a = Trolox equivalent of unknown sample well (nmole) from standard curve

S_v = Sample volume (μL) added into the wells

C = Concentration of antioxidant in sample (mM Trolox equivalents)

2.5.3. α-Amylase inhibitory ability

The α-Amylase inhibitory assay was carried out using the α-Amylase inhibitor screening kit according to the manufacturer's instructions. Briefly, 50 μL of the fermented samples were mixed with 50 μL of diluted α-Amylase enzyme and incubated at room temperature for 10 min. Diluted α-Amylase substrate (50 μL) was added to the enzyme-inhibitor mixture and mixed thoroughly. The optical density was measured at 405 nm under room temperature in kinetic mode for 30 min. Relative percentage inhibition was calculated as shown below:

$$\%\ \text{Relative inhibition} = \left(\frac{Slope\ of\ (EC) - Slopeof\ (T)}{Slope\ of\ (EC)}\right) X\ 100\%$$

Where EC = Enzyme control

T = Test sample

2.5.4. α-Glucosidase inhibitory ability

Fermented samples with α-Glucosidase inhibitory ability were identified using the α-Glucosidase inhibitor screening kit according to the manufacturer's instructions. Briefly, 10 μL of α-Glucosidase was mixed with 10 μL of the samples and incubated in the dark at room temperature for 30 min. An aliquot (20 μL) of α-Glucosidase substrate was added and mixed. Acarbose was used as the standard α-Glucosidase inhibitor and absorbance was measured at 410 nm in the kinetic mode at room temperature for 1 h. Samples whose inhibitory abilities were stronger than fresh beetroot were chosen for further studies. Relative percentage inhibition was calculated as shown below:

$$\%\ \text{Relative inhibition} = \left(\frac{Slope\ of\ (EC) - Slopeof\ (T)}{Slope\ of\ (EC)}\right) X\ 100\%$$

Where EC = Enzyme control

T = Test sample

2.6. Effect of temperature and fermentation time on antioxidant capacity, DPP-IV, α-Amylase, and α-Glucosidase inhibition

The influence of fermentation temperature and fermentation time on antioxidant capacity, DPP-IV and α-Glucosidase inhibition was performed as reported by Yang et al. (Yang et al., 2018) with modifications. Briefly, beetroot samples were fermented either at 30, 37 or 45 °C for 24, 48 or 72 h using the selected lactic acid bacterium. The bacteria growth kinetics was measured at 600 nm using a spectrophotometer and the changes in pH were measured periodically with a pH meter (Mettler Toledo® FiveEasy, Brooklyn, NY). The fermented sample that showed the strongest antioxidant capacity was tested for DPP-IV, α-Amylase and α-Glucosidase inhibitory abilities using the methods earlier described.

2.7. Genetic identification of selected bacterium

Following the manufacturer's instructions, QIAGEN DNeasy PowerSoil Pro Kit was used to extract DNA from the samples and whole genome sequencing was carried out by CosmosID (Germantown, MD, USA). DNA samples were measured using the QuantiFluor® dsDNA System (Promega) chemistry and the GloMax Plate Reader System from Promega. The Nextera XT DNA Library Preparation Kit (Illumina) and IDT Unique Dual Indexes were used to create DNA libraries with a total DNA input of 1 ng. An equal amount of Illumina Nextera XT fragmentation enzyme was used to lyse genomic DNA. The forward and reverse primer sequences used were AGAGTTTGATCCTGGCTCAG and GNTACCTTGTTACGACTT respectively. Each sample received distinct dual indexes, and then 12 cycles of PCR were used to build libraries. DNA libraries were cleaned using Beckman Coulter AMpure magnetic beads and rinsed with QIAGEN EB buffer. Qubit 4 fluorometer and Qubit dsDNA HS Assay Kit were used to quantify DNA libraries. Illumina NovaSeq platform 2x150bp was then used to sequence the libraries. With a read quality trimming threshold of 22 for isolates, raw paired end reads were trimmed and processed using BBDuk. Using SPAdes and the -careful parameter, the trimmed fastqs were put together. CheckM's lineage_wf function was used to assess how complete the built isolate was. In order to assess the phylogenetic placement and single-nucleotide polymorphism (SNP) differences for useful epidemiological conclusions, the assembled contigs were subsequently processed though the CosmosID core genome SNP typing pipeline. Parsnp was used as the core genome aligner in the CosmosID SNP typing pipeline to align the core genomes of various microbial genomes. The phylogenomic link among the genome was then constructed by Parsnp utilizing FastTree2 from the remaining set of core-genome SNPs.

2.8. HPLC-MS analysis of beetroot samples

Chromatographic analysis was carried out as reported by Slatnar et al. (Slatnar, Stampar, Veberic, & Jakopic, 2015) with midifications using HPLC-MS system (Shimadzu, Japan) equipped with a photodiode array (PDA) detector (Shimadzu, Japan) and mass spectrometer (LCMS-2020; Shimadzu, Japan) with an electrospray ionization (ESI) source. The chromatographic separation was conducted using a YMC Pack Pro C18 column (3 × 150 mm; YMC, Japan) at 35 °C and a mobile phase that consisted of 0.1% formic acid water solution (solvent A) and acetonitrile (solvent B) delivered in the 3–50% gradient elution mode at a flow rate of 0.5 mL/min. Mass scans were measured from m/z 50 up to m/z 2000 at a 350 °C interface temperature, 250 °C desolvation line (DL) temperature, ±4500 V interface voltage, and neutral DL/Qarray, using N_2 as nebulizing and drying gas. Mass spectrometry data were acquired in both positive and negative ionization modes. The data were analyzed using LabSolutions liquid chromatography-mass spectrometry (LCMS) software. Compounds were confirmed by comparing UV–Vis spectra, retention times, *m/z* from MS of $[M+H]^+$ and previous reports in literature.

2.9. Statistical analysis

Data were analyzed using Graphpad Prism 9.0. (Graphpad Software, San Diego, USA). All results were presented as mean ± standard deviation (SD) of triplicate analyses. Values were compared using one-way analysis of variance (ANOVA) followed by Tukey's test at $p < 0.05$ significance level.

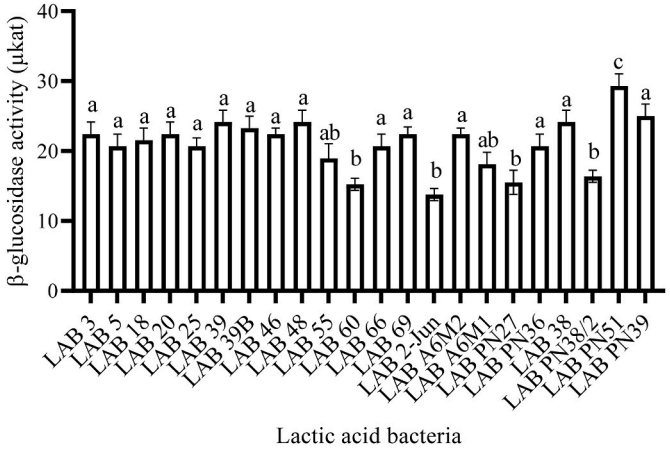


Fig. 1. β-D-glucosidase activity of lactic acid bacteria isolated from fermented foods.
Each bar represents the means of three replicates (n = 3) ± S.D.

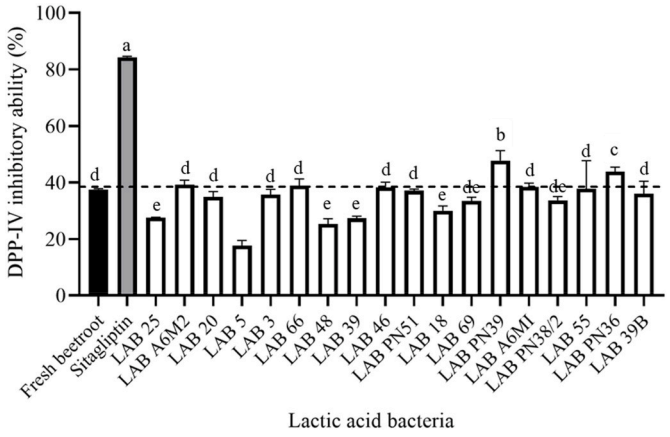


Fig. 2. DPP-IV inhibitory activity of beetroot samples fermented with lactic acid
bacteria compared with the inhibitory ability of fresh beetroot. Each bar represents the
means of three replicates (n = 3) ± S.D. Dotted line indicates the percentage inhibition
of fresh beetroot such that bars taller the dotted line have stronger DPP-IV inhibitory
abilities than fresh beetroot juice. Bars with different alphabets indicates significant
difference ($p < 0.05$).

3. Results and discussion

3.1. Screening for lactic acid bacteria with β-Glucosidase activity

Deglucosylation during fermentation is a known mechanism for improving functional activities during functional food development (Michlmayr & Kneifel, 2014; Ávila et al., 2009). Hence, we selected lactic acid bacteria for beetroot fermentation based on their ability to hydrolyze beta-glycosidic bonds. All the 22 bacteria tested showed β-D-Glucosidase activity to various extents and this agrees with earlier studies that reported that the enzyme is widespread in lactic acid bacteria (Michlmayr & Kneifel, 2014). Meanwhile, the differences in enzyme activities among the strains could be due to strain specificity and differences in bacteria metabolism. In order to select only bacteria with the highest β-D-Glucosidase activities among the 22 isolates, strains that exhibited the least enzyme activities were excluded from further studies (Fig. 1). The remaining 18 bacteria were used for beetroot fermentation and further analysis.

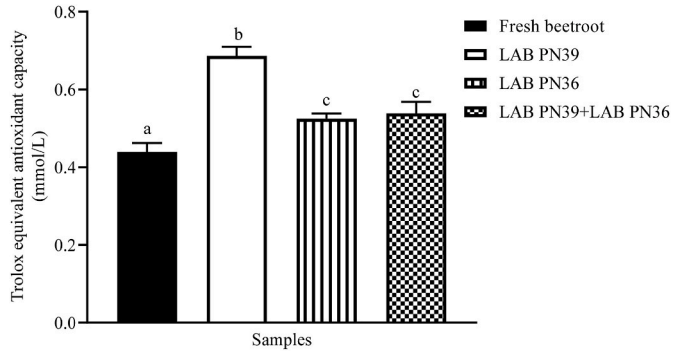


Fig. 3. Total antioxidant capacity of beetroot fermented with lactic acid bacteria
compared with fresh beetroot juice. Each bar represents the means of three replicates
(n = 3) ± S.D. Bars with different alphabets are significantly different ($p < 0.05$).

3.2. Effects of beetroot fermentation on DPP-IV inhibitory ability

Diabetes patients have high plasma DPP-IV activity which hydrolyze glucagon-like peptide-1 (an insulinotropic and glucose-lowering molecule) resulting in impaired glucose metabolism and hyperglycemia (Sarkar, Nargis, Tantia, Ghosh, & Chakrabarti, 2019). Therefore, fermented foods that inhibit DPP-IV activity have potential antidiabetic abilities. Out of the 18 fermented samples tested against DPP-IV in this study, only those fermented with LAB PN36 and LAB PN39 demonstrated inhibitory abilities stronger than that exhibited by fresh beetroot juice (Fig. 2). Specifically, samples fermented with LAB PN39 demonstrated the strongest DPP-IV inhibitory ability of 50 ± 3.5% while samples fermented with LAB PN36 had inhibitory abilities of 43.8 ± 1.6%. However, the DPP-IV inhibitory ability of fresh beetroot was only 37.5 ± 0.3%. This indicates that fermentation with these bacteria significantly improved ($p < 0.05$) the DPP-IV activity of beetroot. The samples fermented with these two bacteria were therefore subjected to further analysis.

3.3. Effects of beetroot fermentation on antioxidant capacity

During diabetes, hyperglycemia activates the electron transport chain which generates large amounts of ROS, impairs beta-cell functions and elevates insulin resistance (Ayer, Fazakerley, James, & Stocker, 2022). However, antioxidants can reduce oxidative stress caused by ROS to alleviate their harmful effects. For this reason, improving the antioxidant capacity of functional foods is imperative for mitigating the disease. In this study, we compared the antioxidant capacity of fresh beetroot juice to juice from beetroot samples fermented with LAB PN39, LAB PN36 and a combination of both bacteria (LAB PN39 + LAB PN36). Interestingly, the antioxidant capacity of fresh beetroot was increased from 0.43 ± 0.07 mmol/L to 0.69 ± 0.04 mmol/L, 0.53 ± 0.03 mmol/L and 0.55 ± 0.04 mmol/L when fermented with LAB PN39, LAB PN36 and LAB PN39 + LAB PN36 respectively (Fig. 3). The increased antioxidant capacity was possibly due to the ability of the bacteria to release bound antioxidant compounds and also to generate bioactive compounds from beetroot during the fermentation process (Zhao et al., 2021). Our results are in agreement with recent studies that have reported that lactic acid bacteria fermentation of food can improve their antioxidant capacities (Madjirebaye et al., 2022; Sandez Penidez, Velasco Manini, LeBlanc, Gerez, & Rollán, 2022). Meanwhile, fermentation with the bacteria cocktail did not result in significant improvements in the antioxidant capacity of the samples relative to single strain fermentation ($p < 0.05$). We therefore proceeded to test the potential of

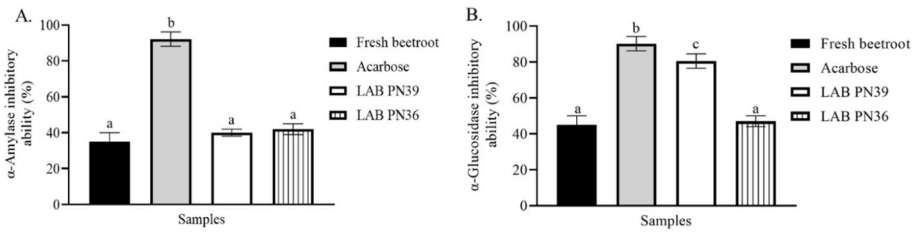


Fig. 4. Inhibition of carbohydrate hydrolyzing enzymes by fermented and unfermented beetroot samples. (A) α-Amylase inhibitory activity of fresh beetroot compared with fermented beetroot. (B) α-Glucosidase inhibitory activity of fresh beetroot compared with fermented beetroot. Bars with the same alphabets are not significantly different ($p < 0.05$).

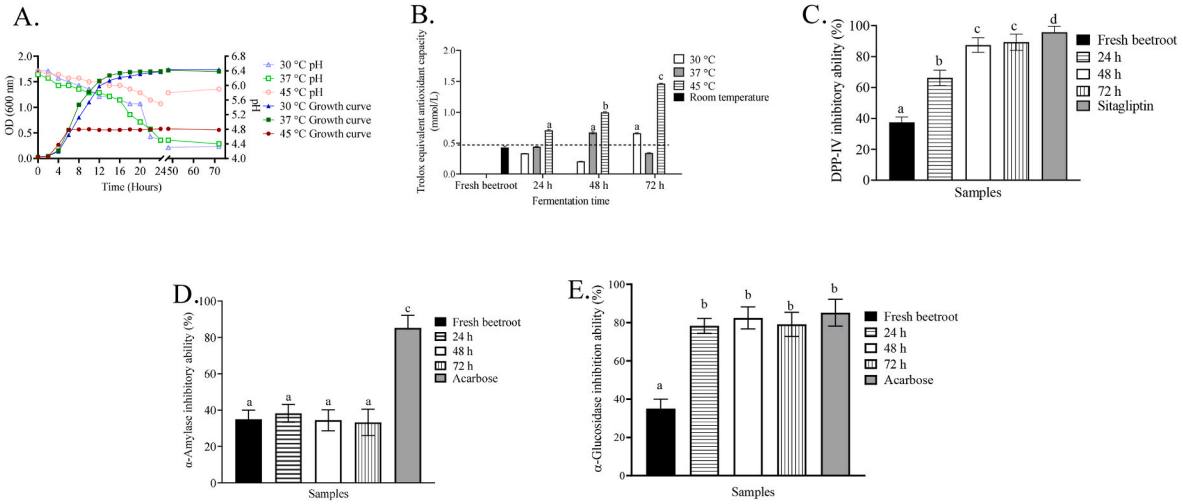


Fig. 5. Effects of fermentation temperature and time on antidiabetic potentials. (A) Microbial growth kinetics under different temperature. (B) Total antioxidant capacity of beetroot fermented under different temperature and time, (C) DPP-IV inhibitory ability of beetroot samples fermented at 45 °C for 24, 48 and 72 h, (D) α-Amylase inhibitory ability of beetroot samples fermented at 45 °C for 24, 48 and 72 h, (E) α-Glucosidase inhibitory ability of beetroot samples fermented at 45 °C for 24, 48 and 72 h.

the samples fermented with LAB PN39 and LAB PN36 to retard dietary glucose release.

3.4. *Effects of beetroot fermentation on carbohydrate hydrolyzing enzymes (α-Amylase and α-glucosidase)*

T2D is particularly characterized by carbohydrate metabolic disorders and hence modulating dietary carbohydrate digestion effectively regulates blood glucose levels (Mills et al., 2022). In the gut, α-Amylases hydrolyze α-1,4 glycosidic bonds of starch into shorter glucose chains during digestion (Kaur et al., 2021). For this reason, inhibiting α-Amylase activity decreases the rate of starch digestion and reduces postprandial hyperglycemia. In this study, there were no significant differences ($p > 0.05$) between the α-Amylase inhibitory abilities of fermented and unfermented samples (Fig. 4a). Indeed, some studies have demonstrated the ability of certain lactic acid bacteria to improve α-Amylase inhibition after fermenting certain foods (Klongklaew et al., 2022; Ujiroghene et al., 2019). Yet the functional activity of a fermented sample would depend on the bacteria species and the substrate used. Meanwhile, since the disaccharides and oligosaccharides released by α-Amylases must be further cleaved to release glucose, we proceeded to test the ability of the fermented samples to inhibit α-Glucosidase activity.

Alpha-Glucosidase hydrolyses α (1 → 4) glycosidic bonds at the non-reducing terminal of carbohydrates to yield α-glucose molecules which increase blood glucose levels after intestinal absorption (Akmal & Wadhwa, 2022). Inhibiting the enzyme activity would therefore delay glucose absorption thereby moderating postprandial hyperglycemia. Among the 2 fermented beetroot samples tested, the sample fermented with PN39 demonstrated a significantly improved α-Glucosidase inhibitory ability of 80.5% (Fig. 4b) compared to fresh beetroot ($p = 0.01$). Takács-Hájos and Vargas-Rubóczki (Takács-Hájos & Vargas-Rubóczki, 2022) have shown that beetroot contains high levels of polyphenolic compounds which are strong α-Glucosidase inhibitors (Cenobio-Galindo et al., 2019) and this could account for the inhibitory ability of fresh beetroot juice. Meanwhile, the ability of PN39 fermented samples to more strongly inhibit α-Glucosidase agrees with a study by Zahid et al. (Zahid et al., 2022) which demonstrated that fermentation of polyphenol-rich foods with lactic acid bacteria can boost the α-Glucosidase inhibitory ability of the final product.

3.5. *Effects of temperature and fermentation time on antioxidant capacity, DPP-IV, α-Amylase and α-Glucosidase inhibition*

Bacteria growth and metabolism are influenced by fermentation temperature and most lactic acid bacteria show optimum growth at temperatures between 30 °C and 45 °C (Yang et al., 2018). Also most studies that have investigated the effects of fermentation time on the antidiabetic potentials of artificially inoculated food samples have done so within 24 h–72 h (Fujita, Sarkar, Genovese, & Shetty, 2017; Klongklaew et al., 2022; Ramakrishna, Sarkar, Dogramaci, & Shetty, 2021). Hence, we investigated the impact of these fermentation conditions on the antioxidant capacity of PN39 fermented beetroot since fermented products from this strain showed the strongest antidiabetic potentials. As shown in Fig. 5A, the bacterium demonstrated similar growth kinetics and reached stationary phase after 18 h when grown at 30 °C and 37 °C. At 45 °C however, stationary phase was reached after 5 h of fermentation. To survive heat stress, lactic acid bacteria possess thermosensors such as CtsR which detect temperature changes and regulate microbial replication leading to stagnant or slow growth during high temperatures (Darsonval, Julliat, Msadek, Alexandre, & Grandvalet, 2018). Also, they excrete catabolic intermediates such as organic acids

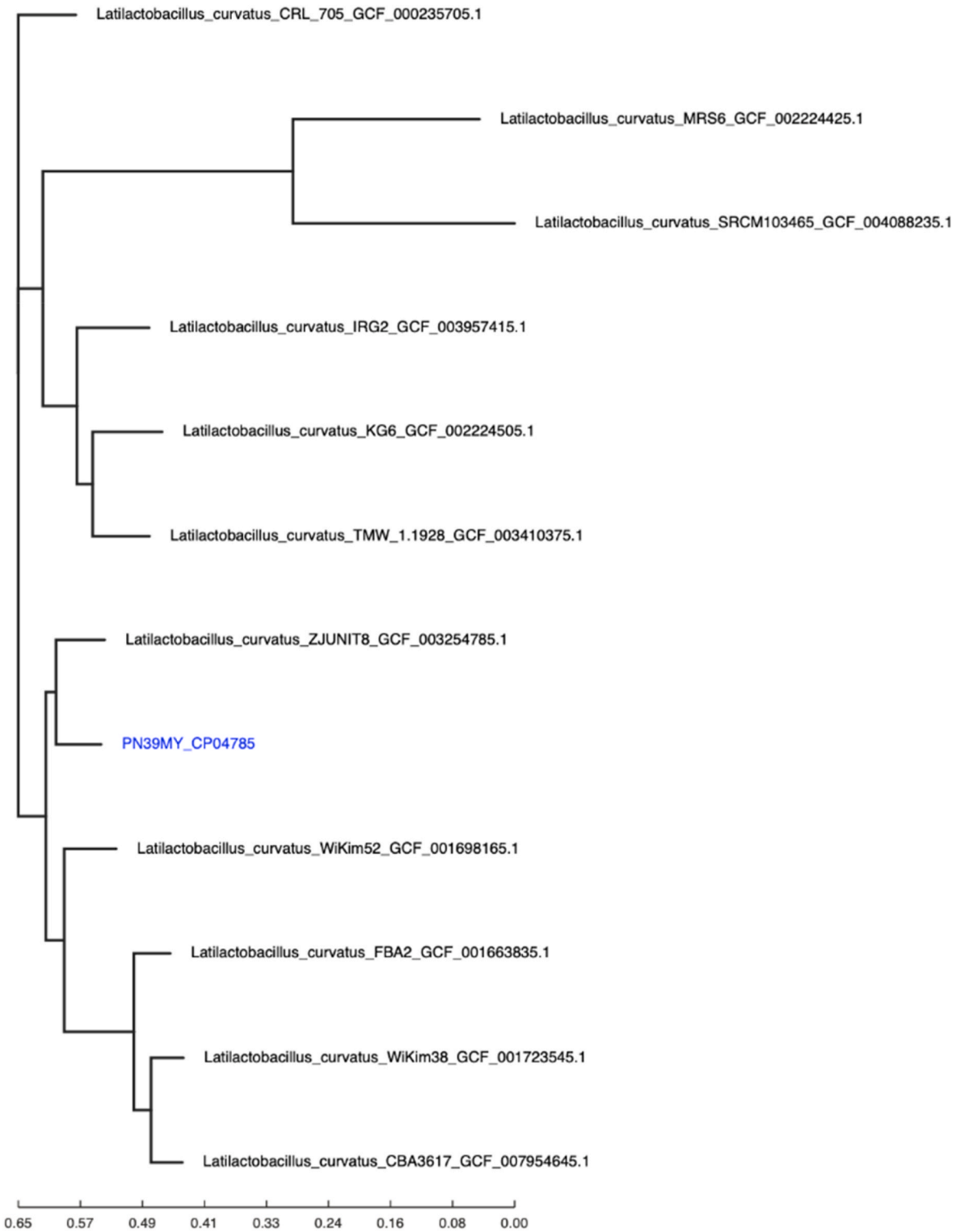
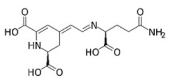
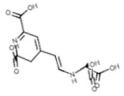
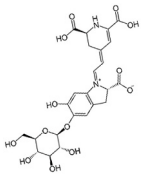
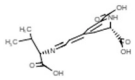
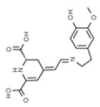
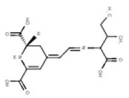
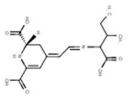
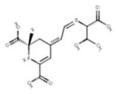
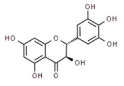
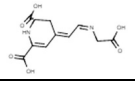


Fig. 6. Phylogenetic tree of LAB PN39MY bacteria. FastTree2 was used to reconstruct the phylogenetic relationship from the core-genome SNPs. The GenBank accession numbers are listed.

into the environment to reduce heat stress (Frank & Evolution, 2020). These factors may account for the growth kinetic of the bacterium at 45 °C and the continuous reduction in sample pH from 6.3 to 5.5 at the 24th hour of fermentation though stationary phase was reached at the 5th hour. Fermentation at 30 °C significantly increased ($p = 0.02$) the antioxidant capacity of beetroot only when the sample was fermented for 72 h (Fig. 5B). On the other hand, samples fermented at 37 °C had increased antioxidant capacity from 0.43 ± 0.02 mmol/L (in fresh beetroot) to 0.0.69 ± 0.01 mmol/L after 48 h of fermentation. However, the antioxidant capacity decreased to 0.34 ± 0.01 mmol/L when fermentation time was increased to 72 h. The decrease in antioxidant capacity could be because some of the antioxidant compounds generated at the 48th h served as energy sources for microbial growth or as substrates for generating new compounds. Meanwhile, fermenting beetroot

Table 1
Chromatographic and mass spectrometric data of analyzed pigments in red beetroot juice before and after fermentation with *L. curvatus* PN39.

Peak No.	Compounds	Retention time (min)	UV–vis maximum (nm)	m/z [M + H]+	Peak area	Structure
Unfermented sample						
1	Vulgaxanthin I	3.5	471	340	1819461	
2	Vulgaxanthin I	4.8	471	340	786804	
3	Glutamic acid-betaxanthin	5.5	471	341	67467	
4	Glutamic acid-betaxanthin	6.8	468	341	58325	
5	Unknown	8	457	297	25215	
6	Indicaxanthin	9.5	479	309	52930	
7	Betanin	10.5	532	551	1474903	
8	Betanin	11.5	531	551	938603	
9	Betanin	12.5	532	551	245988	
10	Unknown	13	526	539	167580	
11	Valine-isobx	14	472	311	39128	
12	Valine-isobx	14.7	472	311	29305	
13	3-methoxytyramine-betaxanthin	17	412	361	42810	
14	Isoleucine-isobx	18.6	472	325	54402	
15	Isoleucine-bx/Leucine-isobx	19.5	472	325	59165	
LAB PN 39 fermented sample						
1	Threonine-betaxanthin	12	467	313	6140	
2	Unknown	13	476	313	14673	
3	Dihydromyricetin	14.5	476	321	71422	
4	Glycine-betaxanthin	15	461	267	53630	

at 45 °C increased the antioxidant capacity to 0.707 ± 0.01 mmol/μL after 24 h. The antioxidant capacity further increased to 1.00 ± 0.01 nmol/μL and 1.46 ± 0.01 mmol/μL after fermentation for 48 h and 72 h respectively. Our results agree with earlier studies that reported that fermentation temperature and time have significant effects on antioxidant activities of the final product (Liu et al., 2020). Interestingly, the antioxidant capacity of samples fermented at 45 °C for 24 h were not significantly different ($p = 0.21$) from those fermented at 37 °C for 48 h and 30 °C for 72 h. This indicates that increasing fermentation temperature increased antioxidant activity and decreased fermentation time.

Since fermentation at 45 °C demonstrated the highest antioxidant capacity, we maintained the fermentation temperature but varied the fermentation time to determine the impact of fermentation time on DPP-IV, α-Amylase and α-Glucosidase inhibitory activities. As shown in Fig. 5C, DPP-IV inhibition increased with fermentation time when temperature was held at 45 °C. Inhibition increased from 37.4% (in the fresh juice) to 66.3% after 24 h fermentation and further increased to 87.45 ± 3.2% after 48 h. However, there was no significant difference ($p > 0.05$) in the inhibitory abilities of samples fermented for 48 h and 72 h.

Meanwhile, fermenting beetroot at 45 °C up to 72 h had no significant effects ($p > 0.05$) on the α-Amylase inhibition activities (Fig. 5D). Yet, although fermenting beetroot improved its α-Glucosidase inhibitory abilities relative to fresh beetroot, extending the fermentation time beyond 24 h had no significant effect ($p > 0.05$) on its inhibitory effect (Fig. 5E).

3.6. Genetic identification of LAB PN39 strain

Comparative analysis using Cosmos ID bacteria database showed that LAB PN39 was closest to *Latilactobacillus curvatus* sowing a core genome coverage of 71.9%. The strain formed a separate cluster with *Latilactobacillus curvatus_ZJUNIT8_GCF_003254785.1* (Fig. 6). We therefore named the isolate as *Latilactobacillus curvatus* strain *PN39MY. The whole genome sequence of the bacterium was deposited at NCBI with* accession number *NZ_JARGYE010000000.*

3.7. Effects of high temperature fermentation on red beetroot betalains

Generally, two betacyanins (betanin and isobetanin) and seven betaxanthins (gulgaxanthins I, glutamic acid-betaxanthin (vulgaxanthins II), indicaxanthin, valine-isobetaxanthin, 3-methoxytyramine-betaxanthin, isoleucine-betaxanthin and leucine-isobetaxanthin) were detected in the fresh beetroot with vulgaxanthin I being the most abundant followed by betanin (Table 1). After fermentation with *L. curvatus* PN39, theonine-betaxanthin and glycine-betaxanthin were the only betaxanthins present in the fermented samples but no betacyanins were detected. This is possibly because the fermentation process might have converted these compounds into other molecules (Choińska et al., 2022). Meanwhile, the loss of betanin in the fermented sample could be due to the high fermentation temperature and this agrees with earlier studies that reported that fermenting beetroot under high temperature drastically reduces betalain levels up to about 88% (Choińska et al., 2022; Czyżowska et al., 2006; Sawicki & Wiczkowski, 2018). However, the most abundant compound in the fermented sample was dihydromyricetin which is known to strongly inhibit ROS, DPP-IV (Wu et al., 2022) and α-Glucosidase activities (Mi et al., 2021). Indeed, the anti-hyperglycemic effects of dihydromyricetin in type 2 diabetes animal models is well documented (Yao et al., 2021) and hence, the presence of dihydromyricetin in the fermented sample may have contributed immensely to the strong antidiabetic potentials displayed in this study.

4. Conclusions

In this study, we have demonstrated that *Latilactobacillus curvatus* PN39MY isolated *from Lithuanian fermented vegetables* is capable of fermenting red beetroot to improve its antidiabetic properties. Specifically, we have demonstrated that fermenting beetroot with *L. curvatus* PN39MY strongly improved antioxidant capacity, α-Glucosidase and DPP-IV inhibitory abilities. Fermentation with this strain at 45 °C for 72 h further improved its antidiabetic potentials probably due to the generation of high amounts of the antidiabetic compound dihydromyricetin. Further studies are however required to prove the antidiabetic ability of the fermented product in diabetic animal models. Also, cell line studies would be required to establish the potential molecular mechanisms by which the fermented sample may elicit its effects.

CRediT authorship contribution statement

Eric Banan-Mwine Daliri: Conceptualization, Formal analysis, Writing – original draft. **Toma Balnionytė:** Formal analysis. **Jonita Stankevičiūtė:** Formal analysis. **Eglė Lastauskienė:** critical review of the manuscript, Writing – review & editing. **Rolandas Meškys:** critical review of the manuscript, Writing – review & editing. **Aurelijus Burokas:** Conceptualization, Resources, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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