scientific reports

Check for updates

OPEN

Characterization and immune-modulatory roles of branched glucan and acetylated gluco-oligosaccharides produced by glucansucrase 40 from *Leuconostoc mesenteroides* YTU 40

Sevda Dere[1], Hümeyra İspirli[2], Kader Korkmaz[1], Yunus Emre Tuncil[3], Osman Sagdic[1], Arjan Narbad[4], Mutamed Ayyash[5]✉ & Enes Dertli[6]

Glucansucrases (GS) are functional enzymes for the production of distinct glucan and gluco-oligosaccharide structures with potential bioactive characteristics. In this study, an active glucansucrase (GS40) was identified within the genome of *Leuconostoc mesenteroides* YTU 40 that was further cloned, heterologously expressed and biochemically characterised. GS40 was capable of production of a branched dextran with ~ 20% (1→3)-α-D-Glc residues detected by ^{1}H and ^{13}C NMR analysis. Acceptor reactions of GS40 with maltose and cellobiose were performed and distinct maltose/cellobiose derivative acetylated gluco-oligosaccharides were produced detected by TLC, NMR and LC-MS analysis. The bioactive characteristics of branched dextran and gluco-oligosaccharides were tested by determination of their immune-modulatory roles in terms of triggering IL-12, IL-10, IL-4 and TNF-α cytokine levels. These findings can be important to unveil the role of glucansucrases for the production of gluco-oligosaccharides that can be used as functional ingredients for food and other industries.

In recent years, interests in oligosaccharides with different structures has been increasing due to their functional properties positively affecting the homeostasis of host[1]. So far studies have focused on oligosaccharides such as fructooligosaccharides (FOS), galactooligosaccharides (GOS) and xylooligosaccharides[2] and recently gluco-oligosaccharides produced by glucansucrases are of interest due to their potential functionalities as well as their suitability for carbohydrate engineering[3–6]. The production methodologies for distinct oligosaccharides include extraction, polymer hydrolyzation, enzymatic and chemical methods and environmentally friendly methodologies such as utilisation of specific enzymes have gained special position, as the other methods require longer steps and a wide variety of by-products can be formed in the reaction environment. In this sense, glucansucrases (GS) that can be encoded in the genomes of Lactic Acid Bacteria (LAB)[7] are such good examples as glucansucrases can utilise sucrose as the donor and in the presence of an acceptor molecule such as distinct sugars, novel oligosaccharides can be formed in an environmentally friendly way with high efficiency and purity[8–10]. So far several glucansucrases from different LAB strains were shown to be effective glucansucrases to produce distinct gluco-oligosaccharides using maltose, cellobiose, raffinose, mellibose, and lactose as well as other sugars as acceptor molecules[4,6,11–14]. These reports demonstrated the transfer of (1→4)-α-D-Glc, (1→2)-α-D-Glc, (1→3)-α-D-Glc and (1→6)-α-D-Glc units to the acceptor sugars during the elongation of the oligosaccharide chain and both the structural conformation of the glucansucrase, type of the acceptor sugar as well as the donor: acceptor ratio can be effective determinants for the final structural characteristics of the

[1]Chemical and Metallurgical Engineering Faculty, Food Engineering Department, Yildiz Technical University, Istanbul, Turkey. [2]Engineering Faculty, Food Engineering Department, Bayburt University, Bayburt, Turkey. [3]Engineering Faculty, Food Engineering Department, Necmettin Erbakan University, Konya, Turkey. [4]Quadram Institute Bioscience, Gut Health and Microbiome Institute Strategic Programme, Norwich, UK. [5]Department of Food Science, College of Agriculture and Veterinary Medicine, United Arab Emirates University (UAEU), P.O. Box 15551, Al Ain, UAE. [6]Chemical and Metallurgical Engineering Faculty, Food Engineering Department, İstanbul Technical University, Istanbul, Turkey. ✉email: mutamed.ayyash@uaeu.ac.ae

gluco-oligosaccharides[4,11,13,14]. Following the demonstration of the glucansucrases as efficient enzymes for the formation of gluco-oligosaccharides with high yields as well as with distinct structural features, the bioactive characteristics of these oligosaccharides have gained special interests and research have focused on their prebiotic properties[4,5,15]. Another important potential beneficial role of these oligosaccharides might be their function as immune-modulatory agents as different studies demonstrated the role of both FOS and GOS as well as other type of oligosaccharides as immune-stimulatory agents[16–18]. More studies are required to demonstrate the potential immune-modulatory functions of gluco-oligosaccharides produced by the glucansucrases. From these perspectives in this study, a novel glucansucrase gene was identified within the genome of *Leuconostoc mesenteroides* AP-40 as a bee pollen isolate[14]. This gene (GS40) was cloned, heterologously expressed and biochemically characterised. At first structural characterisation of glucan produced by GS40 was applied by ^{1}H and ^{13}C NMR analysis. In terms of gluco-oligosaccharide production, acceptor reactions of GS40 using maltose and cellobiose as acceptor molecules were performed and maltose/cellobiose derivative gluco-oligosaccharides were produced. Formation of gluco-oligosaccharides was monitored by TLC analysis which was followed by structural characterisation using NMR analysis. For the determination of the final length of the gluco-oligosaccharides, LC-MS analysis was applied. Finally, the immunomodulatory functions of maltose/cellobiose derivative gluco-oligosaccharides were tested in vitro, using Caco-2 cells and pro-inflammatory TNF-α, IL-12 and anti-inflammatory IL-10 and IL-4 cytokine levels were measured following the gluco-oligosaccharide application. This study may help us to understand the potential role of distinct glucansucrases for the production of novel gluco-oligosaccharides with bioactive characteristics which can be further evaluated for food and pharmaceutical industries.

Materials and methods

Chemicals and reagents

For the cloning and expression of the glucansucrase gene, aLICator Ligation Independent Cloning (LIC) and Expression Kit was used and obtained from Thermo Scientific (USA). Sucrose, maltose and cellobiose were obtained from Sigma-Aldrich (Steinheim, Germany). Chemicals used for the other analysis were all purchased from Sigma-Aldrich (Steinheim, Germany) and were analytically grade.

Bacterial strains, plasmids, media, and growth conditions

Leuconostoc mesenteroides YTU 40, was previously isolated from bee pollen and cultivated as described elsewhere[14]. The aLICator Ligation Independent Cloning (LIC) and Expression Kit (Thermo Scientific, USA) was used to clone the GS40 gene from *L. mesenteroides* YTU40 applying the manufacturer's protocol. PCR processes were carried out with Phusion High-Fidelity DNA Polymerase (Thermo Scientific, USA). *E. coli* TOP10 cells were used for transformation in the cloning process. The GS40 gene was cloned into the IPTG-inducible vector pLATE31 and expressed in the host *E. coli* BL21 (DE3). *E. coli* strains were cultivated aerobically in LB broth (100 µg/mL ampicillin) at 37 °C through stirring at 200 rpm.

Molecular techniques and whole-genome sequencing of *L. mesentroides* YTU40

General procedures of DNA extraction, PCR reaction, agarose gel electrophoresis were performed as described previously[19]. Primers were obtained from Sentegen (Ankara, Turkey) and whole genome sequencing of *L. mesentroides* YTU40 was performed using Illumina HiSeq 4000 platform. For this at first genomic DNA was isolated from strain YTU40 using a Promega Maxwell Cultured Cells DNA Extraction Kit (AS1620) and DNA concentration was quantified with a Qubit reagent (Promega) prior to the DNA sequencing. Following the quantification, a DNA library was constructed using a modified version of Illumina's Nextera protocol known as Low Input Transposase Enabled libraries. 947 409 paired-end 150 bp reads were generated using the Illumina HiSeq 4000 platform. Reads were quality trimmed with bbduk (v.38.06) (sourceforge.net/projects/bbmap) using a minimum quality of 3. For long-read sequencing, a DNA library was prepared using the native barcoding genomic DNA expansion 1–12 kit (with EXP-NBD104, EXP-NBD114 and SQK-LSK109, Nanopore, UK) according to the manufacturer's instructions and sequenced using a MiniION platform (Quadram Institute Bioscience, Norwich, UK). To obtain a high-quality closed genome, Proka system was used and the formed contigs were then searched for the presence of a glucansucrase gene using BLAST comparison with other glycoside hydrolase 70 (GH70) enzymes. In the next step, the possible glucansucrace enzyme was identified by performing multiple amino acid sequence alignments with the Clustal Omega Tool (https://www.ebi.ac.uk/Tools/msa/clustalo). ExPASy Compute pI/ Mw (http://web.expasy.org/compute_pi/) was used to determine the theoretical Mw (molecular weight) of the potential glucansucrase.

Cloning and expression of GS40 from *L. mesentroides* YTU40

A putative GS gene was identified in the genome of *L. mesentroides* YTU40 and this gene was submitted to GenBank with the PP922942 accession number. For the construction of the expression cassette, primers GS40_LIC_F (5'-AGAAGGAGATATAACTATGtataaatctgggaaaatgttag-3') and GS40_LIC_R (5'-GTGGTGGTGATGGTGATGGCCttagcttttaatcagctctccag-3') were designed in accordance with the pLATE31 vector suitable for the LIC strategy. A 4428 bp PCR product was obtained using Phusion High-Fidelity DNA polymerase (Thermo Scientific, USA) and this product was then ligated into the pLATE31 vector using the aLICator LIC and Expression Kit (Thermo Scientific, USA). The construct pLATE31-GS40 was transformed into electrocompetent *E. coli* TOP10 cells and positive colonies were confirmed by colony PCR using Dream Taq DNA polymerase (Thermo Scientific, USA) with LIC Forward Sequencing primer 5'-TAATACGACTCA CTATAGGG-3' and LIC Reverse Sequencing primer 5'-GAGCGGATAACAATTTCACACAGG-3'. Plasmids were extracted from *E.coli* TOP10 cells using EZNA Plasmid DNA Mini Kit I (Omega Bio-Tek, USA) and the expression plasmid was transferred to *E. coli* BL21 Star (DE3). Standard IPTG induction methodology was

applied for the expression of GS40 applied at 25 °C for 24 h with 250 rpm agitation. The cell pellets were obtained by centrifugation and kept at -20 °C for overnight. Afterwards, the cells pellets dissolved in 20 mM NaAc (pH 5.5) buffer, and enzyme extraction was carried out with sonication (1 min sonication at 35 kHz with 1 min cooling at -20°C between bursts for 20 min). In the purification step of the GS40 enzyme, the HiSPur Ni-NiTa purification kit (Thermo Scientific, USA) was used.

Production of in vitro glucan using sucrose as substrate with GS40

Prior to the in vitro glucan production, the pH and temperature optima of GS40 was determined using the Dinitrosalicylic acid(DNS) methodology[20]. The relative activity of the enzyme was tested by determining the fructose produced during the reactions at different temperatures between 20 and 60 °C and different pHs 3–9. The reaction environment contained 100 mM sucrose, 1 mM $CaCl_2$, and 50 mM NaAC with 10 µg of enzyme added. For the in vitro glucan production the reaction pH was set to pH 5.5 and reaction was conducted at 30 °C for 1 day. At the end incubation period, the presence of sucrose in the medium was observed by thin layer chromatography (TLC). When the sucrose in the reaction was finished, two volumes of ethanol was added to precipitate the glucan in the medium. The obtained glucan was dialyzed using a 12–14 kDa dialysis membrane and the content was subjected to lyophilisation.

Structural characterisation of glucan YTU 40 by HPLC and NMR spectroscopy analysis

The monomer composition of the pure glucan was determined by HPLC analysis using previously described methodology[21]. Briefly, the lyophilised glucan was hydrolysed by 0.5 M H_2SO_4 application for 12 h at 95 ∘C and this was followed by the neutralization with 4 M NaOH. The hydrolysed sample was then subjected to RID-10 A Refractive Index Detector containing HPLC system (Shimadzu) with the conditions of CARBO sep CHO-682 Pb column, column temperature at 25 ∘C, H_2O as the mobile phase with flow rate of 0.7 mL min^{-1}. The monomeric composition was then identified. For structural analysis, both ^1H and ^{13}C NMR spectroscopy analysis were applied to unveil the glucan structure produced by GS40 enzyme. For this purpose, approximately 35 mg of lyophilized glucan was dissolved in 500 ul D_2O and ^1H and ^{13}C NMR analysis was perfomed with AVANCE III 400 MHz NMR (Bruker BioSpin International AG) at 300 K. The results were analyzed with MestReC.

Acceptor reactions of GS40 with maltose and cellobiose and formation of gluco-oligosaccharides

Gluco-oligosaccharides were produced with the acceptor reactions of GS40 with maltose and cellobiose as the acceptor sugars using previously described methodologies[5,14]. Briefly, reaction mixture was formed with sucrose (0.4 mg mL^{-1}) as the donor and maltose/ cellobiose (0.1 mg mL^{-1}) as acceptors in the reaction medium containing 20 mM sodium acetate and 1 mM $CaCl_2$ at pH 5.2 as well as the GS40. Reactions were performed at 30 °C and conducted up to sucrose depletion followed by TLC analysis[14,22]. Fructose and if required glucose were excluded from the reaction environment using immobilised *Saccharomyces cerevisiae* cells[3] and the oligosaccharides were subjected to lyophilisation.

Characterisation of maltose/cellobiose derivative gluco-oligosaccharides

The structural characterisation of the formed maltose/cellobiose derivative gluco-oligosaccharides was applied by ^1H NMR analysis as described in part 2.6. For the determination of the molecular masses of maltose/cellobiose derivative gluco-oligosaccharides with respect to their DPs, LC/MS/Q-TOF analysis was performed with an Agilent 6530 LC/MS/Q-TOF instrument and details of the analysis was given elsewhere[23] and the analysis was applied in negative mode.

Immunomodulatory roles of maltose/cellobiose derivative gluco-oligosaccharides

The in vitro immunomodulatory functions of glucan as well as maltose/cellobiose derivative gluco-oligosaccharides produced by GS40 were determined by measuring pro-inflammatory TNF-α, IL-12 and anti-inflammatory IL-10, IL-4 cytokine levels in the Caco-2 cell line (human colon adenocarcinoma, ATCC HTB-37). Caco-2 cells were maintained Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, Sigma) with 10% heat disabled fetal bovine serum (Gibco, USA) and %1 Penisilin-Streptomisin (Cytiva, USA) at temperature of 37 °C in 5% CO_2. At 80% confluence, the cells were released with 0.25% trypsin-EDTA (Gibco, USA) and subcultured[18,24]. To determine IL-4, IL-10, IL-12 and TNF-α cytokine levels, cells were grown in 6-well plates at seeding density of 4×10^5 and glucan and maltose/cellobiose derivative gluco-oligosaccharides were applied to the cells 24 h under the same conditions[18]. Cytokine levels were determined for each cytokine using human ELISA kits (SunRed Biotech, China) as recommended in the manufacturer's protocol.

Statistical analysis

Cytokine induction measurements were performed as triplicates and results are expressed as mean ± standard deviations. Cytokine levels were subjected to one-way analysis of variance (ANOVA) using SPSS (Version 22, SPSS Inc, Chicago, IL, USA). Tukey's multiple comparison test was used to compare differences at the $p < 0.05$ significance level. GraphPad Prism® (Version 10, GraphPad Software Inc., La Jolla, CA 92037 USA) statistical package program was used for graphical drawings.

Results and discussion

L. mesentroides YTU40 GS40 nucleotide and protein sequence analysis

A putative glucansucrase gene with a bp of 4428 bp was identified in the genome of *L. mesentroides* YTU40 following the whole genome sequencing. The BLASTn algorithm was used to determine the similarity of the GS40 with the other determined putative GSs and GS40 nucleotide sequence demonstrated a similarity of 98.12%

and 94.81% with the GSs from *Leuconostoc mesenteroides* subsp. *suionicum* strain DSM 20,241 (CP015247.1) and *Leuconostoc mesenteroides* strain KNU-2 (CP089782.1), respectively. In terms of protein similarity, GS40 with a 1475 amino acid length showed 95.57% protein sequence similarity *Leuconostoc mesenteroides* DPC 7261 according to BLASTp similarity test. Comparison of GS40 with biochemically characterised GTA E81 from *Lactobacillus reuteri* E81 (accession no. MH051191, 1767 aa)[19] based on the Clustal Omega tool alignment, revealed the significant level of alterations in the amino acid sequence of GS40, especially in the catalytic region of the GS, suggesting the potential novelty of the GS40.

Structural characterisation of α-glucan produced in vitro by GS40

GS40 was tested for in vitro glucan production and the pH and temperature optima of GS40 were found to be pH 5.5 and 30 °C, respectively. This finding was in accordance with previous observations in comparison to the biochemical nature of other glucansucrases[25,26] although glucansucrases were reported to act at higher pHs as well as higher temperatures[19,27]. Reaction of expressed GS40 was performed with sucrose and the polysaccharide formed was structurally characterised by HPLC and $^{1}H/^{13}C$ NMR spectroscopy analysis. Glucose was the only sugar monomer in the repeating unit structure of the polysaccharide revealing that GS40 was able to produce a glucan type polysaccharide (data not shown). For the chemical structure, as can be seen in Fig. 1A[1], H spectra demonstrated the specific spectral resonances at 3.3 ppm and 5.3 ppm. The characteristic anomeric signals appeared at 4.8 ppm and 5.13 ppm and demonstrating the presence of (1→6)-α-D-Glc units and (1→3)-α-D-Glc units in the glucan structure, respectively[28,29] (Fig. 1A). Another important observation for the ^{1}H spectra was the fact that H-4 signal (appeared to be at ~ 3.40–3.45 ppm) was observed to be at a high intensity which arose from the terminal glucosyl residues revealing the high level of branching linkages (~ 20%)[30–32]. In addition, no peak was observed at around ~ 4.20 ppm in the ^{1}H spectra of glucan 40 which was previously allocated to H-5 signal of [6)-α-D-Glc*p*-(1 → 3)- α-D-Glc*p*-(1→] residue indicated that the branching (1→3)-α-D-Glc residues were all terminal[30]. The ^{1}H spectra of glucan 40 revealed that GS40 was responsible for the production of a highly branched dextran containing ~ 20% (1→3)-α-D-Glc residues. Finally, the ^{13}C NMR spectra of glucan 40 revealed six signals at 98.9 ppm, 73.9 ppm, 72.1 ppm, 70.8 ppm, 70.01 ppm and 65.6 ppm (Fig. 1B) that were corresponded to C-1, C-3, C-2, C-5, C-4 and C-6, respectively[28,31,33]. Overall, these findings revealed that the expressed GS40 was an active novel glucansucrase responsible for the production of branched dextran with ~ 20% (1→3)-α-D-Glc residues. *Leuconostoc mesenteroides* strains are well-known for classical dextran producers containing low levels of (1→3)-α-D-Glc*p* units as branching points (~ 5%)[28] although recent studies also revealed the production of branched dextrans produced by other *Leuconostoc spp*[28,33]. Nevertheless, capability of obtaining a pure GS40 producing a branched dextran from sucrose can be important to produce this novel dextran at high levels without any impurity that can be further used for distinct technological purposes including gluco-oligosaccharide production.

Production of maltose/cellobiose derivative gluco-oligosaccharides by acceptor reactions of GS40

Following the confirmation of the activity of GS40 for the formation of branched dextran with with ~ 20% (1→3)-α-D-Glc residues, GS40 was tested for its acceptor reactions with maltose and cellobiose to produce novel gluco-oligosaccharides with potential bioactive characteristics. As can be seen in Fig. 2, GS40 was used both sugars as acceptors and gluco-oligosaccharides was formed. Similar to our findings recent studies also reported the utilisation of glucansucrases with distinct sugars as acceptors for the formation of gluco-oligosaccharides containing different glycosidic linkages forming the oligosaccharides[4–6,14,19,22,31,34]. At first, the maltose/cellobiose derivative gluco-oligosaccharides were characterised structurally by ^{1}H NMR analysis to understand the glycosidic linkages during the glycosylation process. Figure 3 demonstrates the ^{1}H spectrum of cellobiose derivative gluco-oligosaccharides where modification of cellobiose can be seen with the formation of specific peaks. Importantly, the spectrum of cellobiose derivative gluco-oligosaccharides produced by GS40 was comparable with the previous studies in which two alternansucrases and one dextransucrase were used for the production of the cellobiose-derived oligosaccharides[5,34,35]. The ^{1}H spectra of the GS40 acceptor product with cellobiose was aligned with the spectra of GTF E81 where 12 doublets with chemical shift from 4.40 ppm and 5.45 ppm was reported previously and these doublets were observed between 4.20 ppm to 5.25 ppm for GS40 acceptor products. In accordance with previous reports, this data revealed the α- and β-anomers of the glucose reducing end of the formed oligosaccharides and addition of the α-linked glucose to cellobiose at either the 2-O position or the 6-O position (Fig. 3). This data suggested the formation of (1→6)-α-D-Glc and (1→2)-α-D-Glc during the elongation of the oligosaccharide chain length of cellobiose-derived oligosaccharides produced by GS40. Previously formation of gluco-oligosaccharides containing (1→6)-α-D-Glc and (1→2)-α-D-Glc was also reported for other glucansucrases using cellobiose as the acceptor sugar[5,34,35] suggesting the potential stereochemistry effect for cellobiose as an acceptor molecule. However, more studies are required to identify the function of cellobiose as an acceptor for the formation of gluco-oligosaccharides containing (1→6)-α-D-Glc and (1→2)-α-D-Glc units. The chemical characterisation of the maltose-derived oligosaccharides produced by GS40 acceptor reaction was also applied using ^{1}H NMR analysis. As can be seen in Fig. 4, anomeric region revealed specific peaks demonstrating the modification of maltose following the acceptor reaction with GS40. Importantly, the ^{1}H spectrum of GS40 maltose reaction product was aligned with the spectra of GTFA-ΔN E81 [19] where specific signals for (1→3)-α-D-Glc (δ ~ 5.13) and (1→6)-α-D-Glc (δ ~ 4.69) units were observed revealing the addition of the α-linked glucose to maltose at either the 3-O position or the 6-O position by GS40. Additionally, specific signals at δ ~ 4.93 and δ ~ 4.35 were observed representing the oligosaccharide reducing end Rα and Rβ, respectively[19]. Similar to the maltose acceptor reaction of GTFA-ΔN E81[1], H NMR profile revealed the formation of the Leucrose.

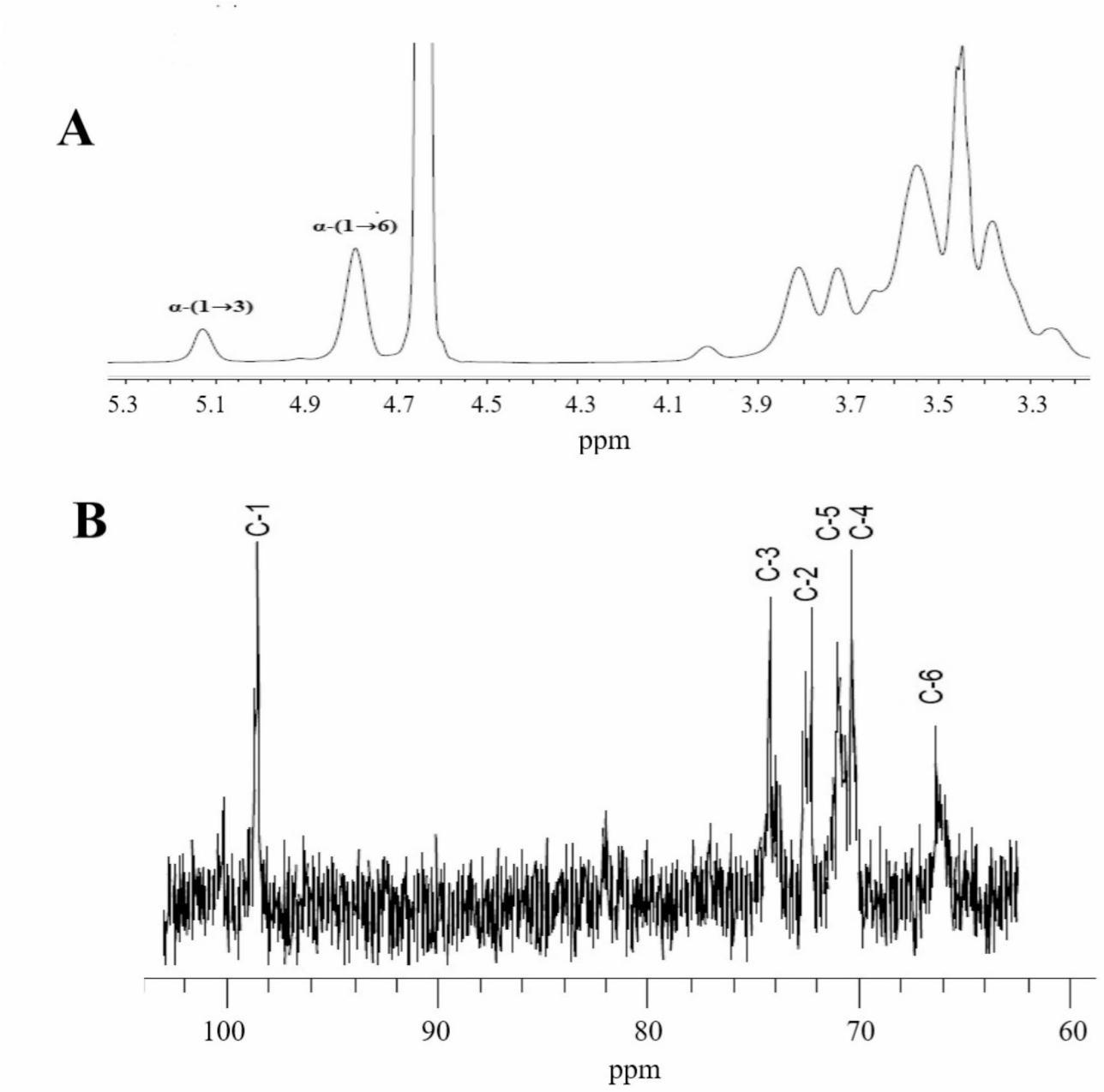

Fig. 1. (**A**) [1]H NMR spectra of branched dextran 40 containing ~ 20% (1→3)-α-D-Glc units produced by GS40 obtained from *Leuconostoc mesenteroides* YTU 40. Specific anomeric peaks at 5.14 ppm and 4.80 ppm which correspond to (1→3)-α-D-Glc and (1→6)-α-D-Glc units, respectively. (**B**) [13]C NMR spectra of branched dextran 40 revealing specific resonances for the (1→3)-α-D-Glc/(1→6)-α-D-Glc units in branched dextran structure.

[α-D-Glc*p*-(1→5)-α-D-Fru*p*] (L:δ ~ 4.8) during the acceptor reaction of GS40 (Fig. 4). In terms of structural features of the maltose-derived glucosoligosaccharides, similar profile was observed for the acceptor reaction of GTFA-ΔN E81 [19] whereas for the acceptor reaction of GTF180-ΔN, (1→2)-α-D-Glc units was observed[36] suggesting the potential role of the distinct glucansucrases and their conformations. Overall these findings demonstrates the formation of cellobiose-derived oligosaccharides containing (1→6)-α-D-Glc and (1→2)-α-D-Glc as well as maltose-derived oligosaccharides containing (1→6)-α-D-Glc and (1→3)-α-D-Glc units by the acceptor reaction of GS40. The final length of the maltose/cellobiose derivative gluco-oligosaccharides was further tested by ESI-MS/MS analysis and Fig. 5A and B demonstrate the total ion chromatogram of the oligosaccharides recorded at $[M]^-$ scan for maltose and cellobiose derivative gluco-oligosaccharides, respectively. As can be seen from Fig. 5B, cellobiose-derived oligosaccharides up to DP 12 was formed by the acceptor reaction of GS40. Previously, formation of cellobiose-derived oligosaccharides up to DP 7 was observed by the activity of distinct glucansucrases[5,34,35] and production of longer oligosaccharides can be important to alter the specific bioactive

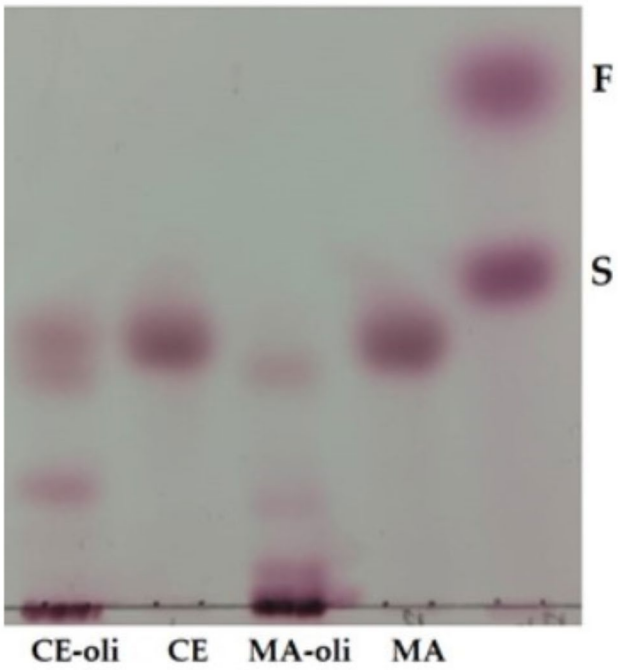

Fig. 2. TLC images of gluco-oligosaccharides produced by the acceptor reactions of GS40 with maltose and cellobiose. F: fructose, S:sucrose, CE: cellobiose, M: maltose, CE-oli/MA-oli: gluco-oligosaccharides produced with the acceptor reaction of cellobiose and maltose.

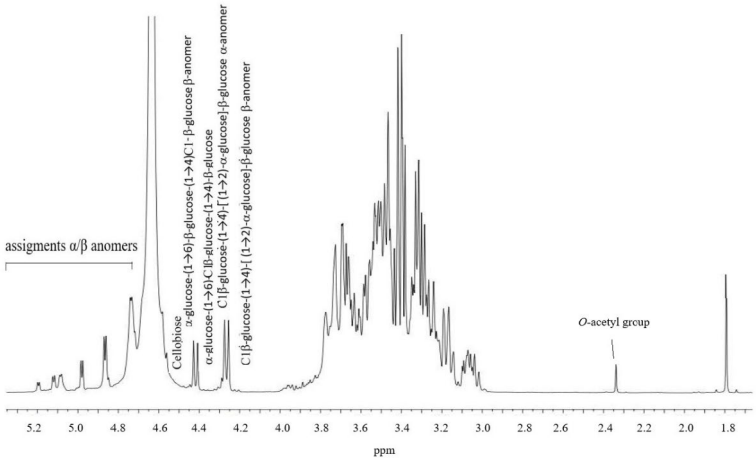

Fig. 3. ^{1}H NMR spectra of cellobiose derivative gluco-oligosaccharides produced by the activity of GS40 demonstrating the presence of (1→2)-α-D-Glc /(1→6)-α-D-Glc units in the oligosaccharide structure. The signal at δ ~2.3 ppm was corresponded to *O*-acetyl groups within the oligosaccharides.

characteristics of these oligosaccharides. In terms of maltose derivative gluco-oligosaccharides, the length of the oligosaccharides was observed to be at DP 9 as the final peak of $[M]^-$ ions was recorded at m/z of 1475.2 corresponding to the analogue of maltononaose (Fig. 5A). This was in accordance with previous observation where the acceptor reaction of GTFA-ΔN E81 with maltose resulted in the formation of gluco-oligosaccharides at DP 9 length[19]. Other studies also reported the formation of similar length[13,37] or shorter gluco-oligosaccharides as DP ≤ 6 [25,26] by the action of distinct glucansucrases suggesting the enzyme specific role of the glucansucrases for the formation of novel gluco-oligosaccharides. Importantly, additional peaks with the corresponding m/z values demonstrated one or two acetylations for both gluco-oligosaccharides (Fig. 5) which was clearly observed

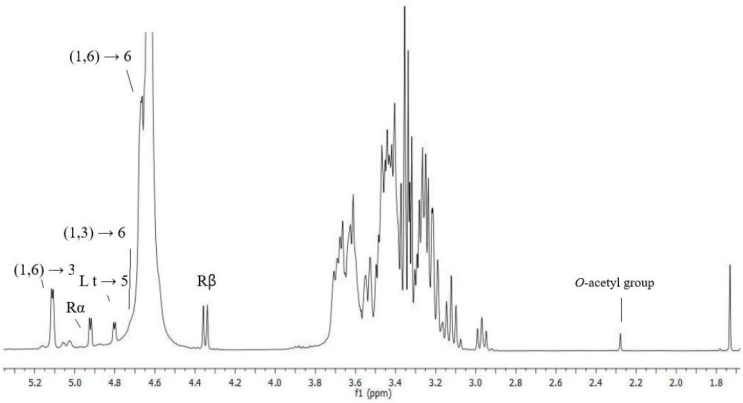

Fig. 4. ^{1}H NMR spectra of maltose derivative gluco-oligosaccharides produced by the activity of GS40 demonstrating the presence of (1→3)-α-D-Glc/(1→6)-α-D-Glc units in the oligosaccharide structure. L: leucrose. The signal at δ ~2.3 ppm was corresponded to *O*-acetyl groups within the oligosaccharides.

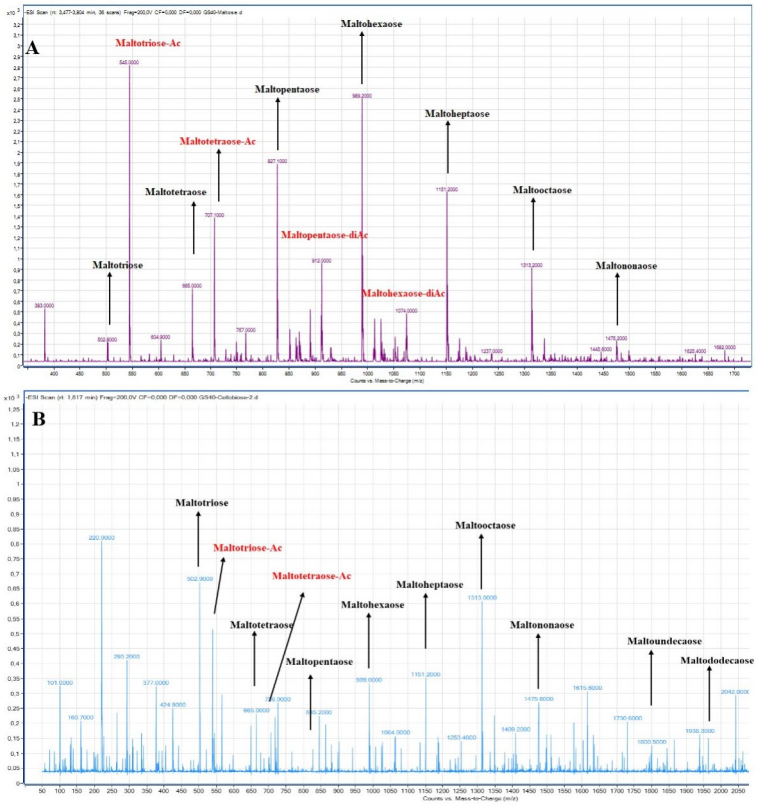

Fig. 5. ESI-MS spectrum of maltose (**A**) and cellobiose (**B**) derivative gluco-oligosaccharides produced by acceptor reactions of GS40 demonstrating the formation of acetylated DP 9 and DP 12 length oligosaccharides, respectively.

with the ESI-MS/MS of maltose derivative gluco-oligosaccharides (Fig. 5A). The presence of the *O*-acetyl groups within the oligosaccharides was also confirmed by the NMR analysis as ^{1}H NMR data of both oligosaccharides demonstrated signals at δ ~2.3 ppm that was previously assigned with the acetyl group of the *O*-acetyl-1,4-linked-β-D-mannan polysaccharides[38]. In addition, for the ^{13}C spectra of both oligosaccharides, clear peaks at δ ~181 ppm (Figure S1) were presented that demonstrated the carbonyl carbon of *O*-acetyl groups in the oligosaccharides as discussed previously[39]. The potential mechanism of acetylation during the glucansucrase acceptor reaction as well as its role for the functionality of oligosaccharides should be further investigated, and

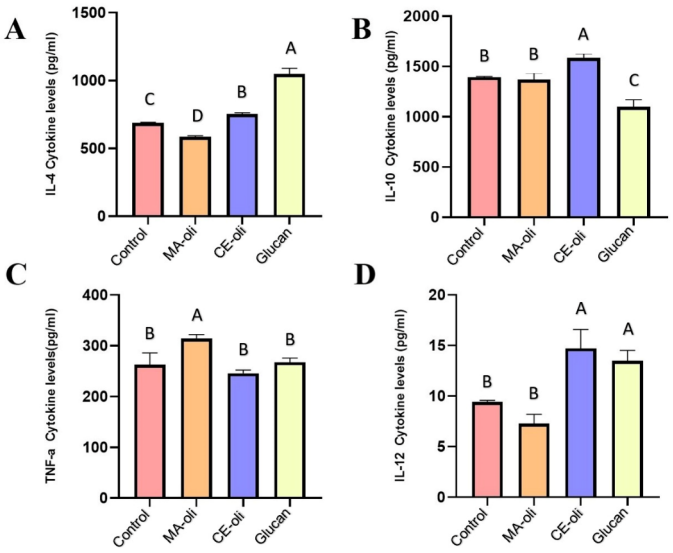

Fig. 6. Immuno-modulatory activity of glucan 40, maltose/cellobiose derivative gluco-oligosaccharides determined under in vitro conditions. Cytokine levels of IL-4, IL-10, IL-12 and TNF-α in Caco-2 cell line after exposure to tested structures were determined in comparison to the control group. Different capital letters show a significant difference ($p < 0.05$) within the tested gluco-oligosaccharides, glucan and control group.

studies are underway to explore the acetylation of oligosaccharides. Overall, GS40 was able to produce acetylated gluco-oligosaccharides up to DP 12 and DP 9 using cellobiose and maltose as the acceptor sugars, respectively.

Immune-modulatory roles of glucan 40 and maltose/cellobiose derivative gluco-oligosaccharides produced by GS40

Recent interests for the bioactive features of the oligosaccharides comprise the potential functionalities of oligosaccharides in terms of their activities as immune-modulatory agents and so far several studies were applied for galacto-oligosaccharides[40,41] and for gluco-oligosaccharides in a lesser extend[18]. In this study, the immune-modulatory roles of glucan 40 and maltose/cellobiose derivative gluco-oligosaccharides produced by GS40 were tested using Caco-2 cells under in vitro conditions by testing the induction levels of IL-12, IL-10, IL-4 and TNF-α cytokines following the 100 µg/ml application of the corresponding compound in comparison to the control. Figure 6A demonstrates the IL-4 cytokine response in Caco-2 cells to glucan 40 and maltose/cellobiose derivative gluco-oligosaccharides, which revealed that a strong induction for IL-4 production was observed for glucan 40 whereas no induction and a relatively low level of induction were detected for maltose and cellobiose derivative gluco-oligosaccharides, respectively. Previously, malto-oligosaccharides containing (α1 → 4) and (α1 → 6) linkages strongly induced the IL-4 cytokine induction[18] but no induction for maltose derivative gluco-oligosaccharides was observed in this study, suggesting the potential role of linkage type between glucose units affecting the IL-4 induction. Strong induction of important anti-inflammatory cytokine IL-4 which could modulate several biological processes by glucan 40 can be important to reveal its bioactive role. This was also the case for the pro-inflammatory cytokine IL-12 as glucan 40 strongly induced its production (Fig. 6D). Previously role of exopolysaccharides for immune-modulation were comprehensively discussed[42,43] but there is a lack of information for the α-glucans and these findings can be important to unveil the immune-modulatory roles of α-glucans. Both glucan 40 and cellobiose derivative gluco-oligosaccharide application resulted in the bipolar induction of the production of pro-inflammatory cytokine IL-12 and anti-inflammatory cytokine IL-4 and this was previously suggested to be a positive factor for the preservation against food allergies as well as infectious diseases tested for weaned piglets[44]. Besides IL-12 and IL-4, only cellobiose derivative gluco-oligosaccharides induced the production of anti-inflammatory cytokine IL-10 (Fig. 6B) as an important cytokine that can be stimulated by probiotic bacteria during probiotic action[45]. This result might also reveal the structure related stimulation potential of gluco-oligosaccharides as well as glucans for distinct cytokines. Similar to our findings previously malto-oligosaccharides produced from maltoheptaose by a glucanotrasferase specifically stimulated IL-10 levels among the tested oligosaccharides[18]. Finally, unlike the other cytokines only maltose derivative gluco-oligosaccharides stimulated the production of pro-inflammatory TNF-α (Fig. 6C) which was indicated to be important stimulant for non-specific immune responses which can be triggered by Lactobacilli exopolysaccharides[46]. In contrast to our data, previously malto-oligosaccharides were reported to be ineffective for the TNF-α induction[18] which again suggests the role of distinct molecular structures for cytokine inductions, and a high level of acetylation in our case could be the reason for this induction. Overall, these findings suggest the importance of the final structural features of the gluco-oligosaccharides as well as α-glucans for the induction of specific immune responses in terms of anti-inflammatory and pro-inflammatory cytokines and more studies

are required to unveil the role of glucansucrase-based oligosaccharides for bioactive functional properties and to explore the role of acetylation for the immune-modulatory functions of these gluco-oligosaccharides.

Conclusion

In this study, GS40 was identified from *L. mesentroides* YTU40, heterologously expressed and biochemically characterised that revealed its function to be responsible for the production of a branched dextran with ~20% (1→3)-α-D-Glc residues. GS40 was also active for its acceptor reactions with cellobiose and maltose and novel acetylated gluco-oligosaccharide structures were formed. GS40 added (1→2)-α-D-Glc or (1→6)-α-D-Glc units to cellobiose during elongation of the oligosaccharides and gluco-oligosaccharides up to DP 12 length were formed. For maltose acceptor reactions, (1→3)-α-D-Glc or (1→6)-α-D-Glc units were added by GS40 and up to DP 9 gluco-oligosaccharides were produced. In terms of bioactive characteristics, both maltose/cellobiose derivative gluco-oligosaccharides and glucan 40 as a branched dextran formed specific immune-responses under in vitro conditions, resulted in different induction levels for IL-12, IL-10, IL-4 and TNF-α cytokines. Identification of novel glucansucrases and testing these enzymes for production of α-glucans as well distinct gluco-oligosaccharides with bioactive characteristics can be important for food industry as these compounds can be used for functional food production strategies.

Data availability

The sequence of glucansucrase YTU 40 (GS40) is available at GenBank with the PP922942 accession number.

Received: 18 November 2024; Accepted: 27 March 2025
Published online: 03 April 2025

References

1. Borewicz, K. et al. Metatranscriptomic analysis indicates prebiotic effect of Isomalto/malto-polysaccharides on human colonic microbiota in-vitro. *Sci. Rep.* **14**, 18866 (2024).
2. Singh, S. P., Jadaun, J. S., Narnoliya, L. K. & Pandey, A. Prebiotic oligosaccharides: Special focus on fructooligosaccharides, its biosynthesis and bioactivity. *Appl. Biochem. Biotechnol.* **183**, 613–635 (2017).
3. Hu, Y., Winter, V., Chen, X. Y. & Gänzle, M. G. Effect of acceptor carbohydrates on oligosaccharide and polysaccharide synthesis by dextransucrase DsrM from Weissella Cibaria. *Food Res. Int.* **99**, 603–611. https://doi.org/10.1016/j.foodres.2017.06.026 (2017).
4. İspirli, H., Bowman, M. J., Skory, C. D. & Dertli, E. Synthesis and characterization of Bifidogenic raffinose-derived oligosaccharides via acceptor reactions of glucansucrase E81. *LWT* 147, 111525, (2021). https://doi.org/10.1016/j.lwt.2021.111525
5. İspirli, H., Bowman, M. J., Skory, C. D. & Dertli, E. Synthesis and characterization of cellobiose-derived oligosaccharides with bifidogenic activity by Glucansucrase E81. *Food Bioscience*. **44**, 101388 (2021).
6. İspirli, H. & Dertli, E. Production of mannose-containing oligosaccharides by Glucansucrase E81 and determination of their functional characteristics. *Biocatal. Biotransform.* **38**, 202–209. https://doi.org/10.1080/10242422.2019.1661384 (2020).
7. Surve, S., Shinde, D. B. & Kulkarni, R. Isolation, characterization and comparative genomics of potentially probiotic Lactiplantibacillus plantarum strains from Indian foods. *Sci. Rep.* **12**, 1940 (2022).
8. Lee, S., Park, J., Jang, J. K., Lee, B. H. & Park, Y. S. Structural analysis of gluco-oligosaccharides produced by leuconostoc lactis and their prebiotic effect. *Molecules* **24**, 3998 (2019).
9. Sanz, M. L., Côté, G. L., Gibson, G. R. & Rastall, R. A. Prebiotic properties of alternansucrase maltose-acceptor oligosaccharides. *J. Agric. Food Chem.* **53**, 5911–5916 (2005).
10. Yang, Y. et al. Reuteransucrase-catalytic kinetic modeling and functional characteristics for novel prebiotic gluco-oligomers. *Food Funct.* **11**, 7037–7047 (2020).
11. Côté, G. L., Holt, S. M. & Miller-Fosmore, C. (ACS, (2003).
12. Côté, G. L., Cormier, R. S. & Vermillion, K. E. Glucansucrase acceptor reactions with D-mannose. *Carbohydr. Res.* **387**, 1–3 (2014).
13. Côté, G. L., Sheng, S. & Dunlap, C. A. Alternansucrase acceptor products. *Biocatal. Biotransform.* **26**, 161–168 (2008).
14. İspirli, H. & Dertli, E. Production of lactose derivative hetero-oligosaccharides from Whey by Glucansucrase E81 and determination of prebiotic functions. *Lwt* **137**, 110471 (2021).
15. Bivolarski, V., Vasileva, T., Gabriel, V. & Iliev, I. Synthesis of glucooligosaccharides with prebiotic potential by Glucansucrase URE 13–300 acceptor reactions with maltose, raffinose and lactose. *Eng. Life Sci.* **18**, 904–913 (2018).
16. Costa, G. T., Vasconcelos, Q. D. & Aragão, G. F. Fructooligosaccharides on inflammation, immunomodulation, oxidative stress, and gut immune response: A systematic review. *Nutr. Rev.* **80**, 709–722 (2022).
17. Chu, H., Tao, X., Sun, Z., Hao, W. & Wei, X. Galactooligosaccharides protects against DSS-induced murine colitis through regulating intestinal flora and inhibiting NF-κB pathway. *Life Sci.* **242**, 117220 (2020).
18. İspirli, H., Şimşek, Ö., Skory, C., Sağdıç, O. & Dertli, E. Characterization of a 4, 6–α–glucanotransferase from Lactobacillus reuteri E81 and production of malto-oligosaccharides with immune-modulatory roles. *Int. J. Biol. Macromol.* **124**, 1213–1219 (2019).
19. İspirli, H. et al. Characterization of a Glucansucrase from Lactobacillus reuteri E81 and production of malto-oligosaccharides. *Biocatal. Biotransform.* **37**, 421–430 (2019).
20. Kim, Y. M. et al. Purification and characterization of a novel Glucansucrase from leuconostoc lactis EG001. *Microbiol. Res.* **165**, 384–391. https://doi.org/10.1016/j.micres.2009.08.005 (2010).
21. Yilmaz, M. T., Ispirli, H., Taylan, O., Alamoudi, M. & Dertli, E. Bioactive and technological properties of an α-D-glucan synthesized by Weissella Cibaria PDER21. *Carbohydr. Polym.* **285**, 119227 (2022).
22. İspirli, H., Kaya, Y. & Dertli, E. Bifidogenic effect and in vitro Immunomodulatory roles of melibiose-derived oligosaccharides produced by the acceptor reaction of Glucansucrase E81. *Process Biochem.* **91**, 126–131 (2020).
23. Song, L. et al. Leuconostoc citreum SK24.002 Glucansucrase: Biochemical characterisation and de Novo synthesis of α-glucan. *Int. J. Biol. Macromol.* **91**, 123–131. https://doi.org/10.1016/j.ijbiomac.2016.05.019 (2016).
24. Paolillo, R., Romano Carratelli, C., Sorrentino, S., Mazzola, N. & Rizzo, A. Immunomodulatory effects of Lactobacillus plantarum on human colon cancer cells. *Int. Immunopharmacol.* **9**, 1265–1271. https://doi.org/10.1016/j.intimp.2009.07.008 (2009).
25. Miao, M. et al. Characterisations of Lactobacillus reuteri SK24.003 Glucansucrase: Implications for α-gluco-poly- and oligosaccharides biosynthesis. *Food Chem.* **222**, 105–112. https://doi.org/10.1016/j.foodchem.2016.12.015 (2017).
26. Song, L. et al. Leuconostoc citreum SK24. 002 Glucansucrase: Biochemical characterisation and de Novo synthesis of α-glucan. *Int. J. Biol. Macromol.* **91**, 123–131 (2016).
27. Pijning, T. et al. Biochemical and crystallographic characterization of a Glucansucrase from Lactobacillus reuteri 180. *Biocatal. Biotransform.* **26**, 12–17. https://doi.org/10.1080/10242420701789163 (2008).

28. Bounaix, M. S. et al. Biodiversity of exopolysaccharides produced from sucrose by sourdough lactic acid bacteria. *J. Agric. Food Chem.* **57**, 10889–10897. https://doi.org/10.1021/jf902068t (2009).
29. Yilmaz, M. T., İspirli, H., Taylan, O., Bilgrami, A. L. & Dertli, E. Structural and bioactive characteristics of a dextran produced by Lactobacillus Kunkeei AK1. *Int. J. Biol. Macromol.* **200**, 293–302 (2022).
30. Meng, X. et al. Biochemical characterization of a GH70 protein from Lactobacillus Kunkeei DSM 12361 with two catalytic domains involving branching sucrase activity. *Appl. Microbiol. Biotechnol.* **102**, 7935–7950 (2018).
31. İspirli, H. et al. Utilisation of an active branching sucrase from Lactobacillus Kunkeei AP-37 to produce techno-functional poly-oligosaccharides. *Int. J. Biol. Macromol.* **236**, 123967 (2023).
32. van Leeuwen, S. S., Leeflang, B. R., Gerwig, G. J. & Kamerling, J. P. Development of a 1H NMR structural-reporter-group concept for the primary structural characterisation of α-D-glucans. *Carbohydr. Res.* **343**, 1114–1119 (2008).
33. Yilmaz, M. T. et al. Characterisation and functional roles of a highly branched dextran produced by a bee pollen isolate leuconostoc mesenteroides BI-20. *Food Bioscience*. **45**, 101330 (2022).
34. Shi, Q. et al. Lactose-and cellobiose-derived branched trisaccharides and a sucrose-containing trisaccharide produced by acceptor reactions of Weissella confusa dextransucrase. *Food Chem.* **190**, 226–236 (2016).
35. Morales, M. A. A., Remaud-Simeon, M., Willemot, R. M., Vignon, M. R. & Monsan, P. Novel oligosaccharides synthesized from sucrose donor and cellobiose acceptor by alternansucrase. *Carbohydr. Res.* **331**, 403–411 (2001).
36. Meng, X. et al. Characterization of the Glucansucrase GTF180 W1065 mutant enzymes producing polysaccharides and oligosaccharides with altered linkage composition. *Food Chem.* **217**, 81–90 (2017).
37. Dobruchowska, J. M. et al. Gluco-oligomers initially formed by the Reuteransucrase enzyme of Lactobacillus reuteri 121 incubated with sucrose and malto-oligosaccharides. *Glycobiology* **23**, 1084–1096 (2013).
38. He, T. B., Huang, Y. P., Wang, X. J., Sheng, J. & Hu, J. M. Structural characterization and biological evaluation of a new O-acetyl-1, 4-linked-β-d-mannan possessed potential application in hydrophilic polymer materials from dendrobium devonianum. *Int. J. Biol. Macromol.* **213**, 328–338 (2022).
39. Liu, L. et al. Acetylation at the O-6 position of t-Glc improved immunoactivity of α-1, 6-glucan from Longan by additionally activating Dectin-1 and CD14 receptors. *Carbohydr. Polym.* **320**, 121199 (2023).
40. Bhatia, S. et al. Galacto-oligosaccharides May directly enhance intestinal barrier function through the modulation of goblet cells. *Mol. Nutr. Food Res.* **59**, 566–573 (2015).
41. Tanabe, S. & Hochi, S. Oral administration of a galactooligosaccharide Preparation inhibits development of atopic dermatitis-like skin lesions in NC/Nga mice. *Int. J. Mol. Med.* **25**, 331–336 (2010).
42. Chaisuwan, W. et al. Microbial exopolysaccharides for immune enhancement: Fermentation, modifications and bioactivities. *Food Biosci.* **35**, 100564 (2020).
43. Hidalgo-Cantabrana, C. et al. Immune modulation capability of exopolysaccharides synthesised by lactic acid bacteria and bifidobacteria. *Probiotics Antimicrob. Proteins*. **4**, 227–237 (2012).
44. Boudry, C. et al. Effect of bovine colostrum supplementation on cytokine mRNA expression in weaned piglets. *Livest. Sci.* **108**, 295–298 (2007).
45. Gourbeyre, P., Denery, S. & Bodinier, M. Probiotics, prebiotics, and synbiotics: Impact on the gut immune system and allergic reactions. *J. Leukoc. Biol.* **89**, 685–695 (2011).
46. Miettinen, M., Vuopio-Varkila, J. & Varkila, K. Production of human tumor necrosis factor alpha, interleukin-6, and interleukin-10 is induced by lactic acid bacteria. *Infect. Immun.* **64**, 5403–5405 (1996).

Acknowledgements

This study was funded by Yıldız Technical University Scientific Research Projects (BAP) with the project number FBI-2022-5143 and United Arab Emirates University (UAEU) via project 12F049. The authors also thank James Choi for technical assistance during the whole genome sequencing. This study is part of Sevda Dere's Master's Thesis.

Author contributions

SD, HI, KK: Writing - Original Draft, Investigation, Validation, Data curation, Visualization; YET, AN, MA, OS: Validation, Funding acquisition, Writing - Review & Editing, MA, ED: Conceptualization, Visualization, Writing - Review & Editing, Funding acquisition, Project administration.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-96324-9.

Correspondence and requests for materials should be addressed to M.A.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by-nc-nd/4.0/.

© The Author(s) 2025