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Growth and *fos*E gene expression in inulin-containing medium of two strains of *Lactobacillus casei* originated from the human intestinal tract

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Abstract. *Authors. 2019. Growth and* fosE *gene expression in inulin-containing medium of two strains of* Lactobacillus casei *originated from the human intestinal tract. Biodiversitas 20: 2758-2763.* Inulin is a fructooligosaccharide consisting of a fructose monomer with β-(1,2) glycosidic linkage. The human colon cannot degrade inulin because it lacks the enzyme that degrades β- (1,2) glycosidic linkage. *Lactobacillus casei* strain AP is able to grow in inulin, *L. casei* strain AF grows gradually. The ability to grow in inulin is proposed by the *fos*E gene, which hydrolyzes β- (1,2) glycosidic linkage. The present study aimed to detect and measure *fos*E gene expression in inulin. Extracellular and intracellular inulin and fructose concentrations were calculated using high-performance liquid chromatography. The reduction of inulin concentration in *L. casei* strain AP was higher than that in *L. casei* strain AF. Extracellular fructose concentration was lower in *L. casei* strain AP because fructose from inulin degradation was transported into the cell. No fructose was detected inside *Lactobacillus* cells. The *fos*E gene was detected in *L. casei* strains AP and AF with a length of 229 bp. *fos*E gene expression was up-regulated 5.9-fold in *L. casei* strain AP and 1.7-fold in *L. casei* strain AF and was three times higher in *L. casei* strain AP than that in *L. casei* strain AF, suggesting that the former metabolizes inulin better than the latter.

Keywords: β-fructosidase, β-glicoside, *fos*E, inulin

INTRODUCTION

Probiotics are bacteria incorporated into food or drinks to increase the health benefits for humans. Milk fermented with probiotic *Lactobacillus casei* strain AP decreases total cholesterol, low-density lipoprotein, and triglyceride in obese individuals (body mass index > 25; Denta 2018). Bacterial species and strains that are commonly used as probiotics are *L. casei* strain Shirota, *L. casei* Zhang (Wang et al. 2013), *Lactobacillus rhamnosus* (Kankainen et al. 2009), *Lactobacillus paracasei*, and *Lactobacillus plantarum* (Buntin et al. 2017). These bacteria need selective nutrition to support their growth, which is described as prebiotic. One example of prebiotics is inulin. Inulin is a fructooligosaccharide (FOS) linked by β- (1,2) glycosidic linkage. Inulin consists of fructans with varying degrees of polymerization (DP) from 2 to 10. Inulin cannot be metabolized by the human colon, which lacks enzymes that degrade β- (1,2) glycoside linkage. Inulin sources are generally from chicory, onion, garlic, and banana, which are abundant in Indonesia. Some *Lactobacillus* strains can grow in inulin, such as *L. casei* strains AP and AG, *L. casei*, and *L. paracasei* (Petrova et al. 2015). Inulin hydrolysis is carried out by the hydrolase family enzyme GH32 such as β-fructocidase, inulinase, and sucrose 6-phosphate hydrolase (Boger et al, 2018).

Inulinase acts to hydrolyze inulin in two ways, namely, exoinulinase and endoinulinase. Exoinulinase hydrolyzes β- (1,2) linkage in terminal residues, releasing fructose as the main product. Fructose is then transported into the cell to be metabolized. Endoinulinase hydrolyzes β- (1,2) linkage at nonterminal residues, releasing short-chain inulin with various DPs (Makras et al, 2005). Short-chain inulin can be transported to bacterial cells using active transporters. Meanwhile, FOS can be degraded inside the cells (intracellular) and outside the cells (extracellular). In *Lactobacillus acidophilus*, *msm* operon consists of *msm*EFGK gene encoding ATP-binding cassette (ABC) transporters and *bfr*A gene encoding intracellular β-fructosidase (Barrangou et al. 2003). In *L. plantarum*, *pts*1BCA is involved in sucrose phosphoenolpyruvate (PEP)-dependent phosphotransferase (PTS; Kleerebezem et al. 2003). *L. casei* strain AP also has the PTS system mannose/fructose/sorbose-specific IID component, ABC transporter, and ABC transporter substrate-binding protein (Widodo et al. 2017). The extracellular enzyme β-fructosidase encoded by *fos* operon consists of *fos*ABCDXE. *Fos*ABCD encodes protein transporters, *fos*X encodes protein regulators, and *fos*E encodes enzymes (Buntin et al. 2017). These genes are induced by oligosaccharides and repressed by its product.

In this study, *L. casei* strain AP, which degrades inulin as sole carbon, and *L. casei* strain AF, which is unable to degrade inulin as a carbon source, were compared and examined. We measured the concentrations of inulin and fructose as the products of inulin hydrolysis both extracellularly and intracellularly to propose enzymes that might be responsible for inulin degradation in *L. casei*. The

corresponding gene fragments for inulin degradation were also amplified, and gene expression was measured.

MATERIALS AND METHODS

Lactobacillus casei strains AP and AF were used in this study. We used MRS and modified MRS containing 10 g/L inulin as sole carbon (called inulin) to grow *L. casei*. Primers for *fos*E gene amplification consist of forward (5′-GGTCAGCAAGCCCTCATTTA-3′) and reverse primer *inu32* (5′-GTTCCGTCGGCTGTCATATT-3′; Aditiyarini 2016). Inulin and fructose standard (Sigma Aldrich) were used as standard fructose and inulin for high-performance liquid chromatography (HPLC) analysis.

Growth of bacteria in inulin

Lactobacillus casei strains AP and AF were cultured in MRS for 24 h at 37°C under micro-aerobic conditions. After 24 h of incubation, the culture was centrifuged at 3000 rpm for 20 min. The pellets were then washed twice with 0.85% NaCl solution and were transferred to inulin. The culture of *L. casei* strains AP and AF was incubated for 24 h at 37°C. Optical density (OD) was measured at 620 nm with a 2 h interval.

Sugar determination using HPLC

Inulin and fructose determination followed the procedure of Widodo et al. (2017), with some modifications. *L. casei* strains AP and AF were harvested at 20 h with a 2 h interval for extracellular analysis and 2, 6, and 12 h for intracellular analysis. Intracellular sugar analysis was carried out using an HPLC instrument with a polyamine column. Stock solutions for calibration of standard inulin and fructose were 12.5, 25, 50, 100, 200, 500, and 1000 ppm. The calibration equations for extracellular inulin, extracellular fructose, intracellular inulin, and intracellular fructose are presented in Equations 1 to 4, respectively:

$y = 14590x + 76268$ (1)
$y = 39075x + 118250$ (2)
$y = 363.57x-65077$ (3)
$y = 139.97x-51204.$ (4)

These equations were used to calculate the sugar concentration of the sample (x) with peak area data (y).

DNA isolation and *fos*E gene amplification

Lactobacillus casei strains AP and AF were grown in MRS and inulin and fermented overnight. The DNA isolation procedure was conducted by following the Presto Mini gDNA bacteria kit Geneaid. *fos*E gene amplification was performed using KAPA taq ReadyMix PCR. This consisted of 5 µL Master Mix 2× KAPA Taq ReadyMix, 1 µL primer forward, 1 µL primer reverse, 1 µL template DNA, and 2 µL water-free DNase. PCR cycling conditions were as follows: 95°C for 5 min preliminary denaturation, followed by 25 cycles of denaturation at 95°C for 30 s, amplification at 59°C for 30 s, elongation at 72°C for 1.5 min, and final elongation at 72°C for 7 min. DNA visualization was done using 2% gel agarose with 4 µL/100 mL ethidium bromide.

Sequencing and bioinformatic analysis

*fos*E was sequenced, and the sequence was aligned with the nucleotide database using Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenic tree was built using software MEGA 7 with maximum likelihood algorithm.

RNA isolation and cDNA synthesis

Lactobacillus casei strains AP and AF were inoculated into MRS and inulin for 18 h. Total RNA was isolated using Presto Mini RNA Bacteria Kit Quick Protocol (Geneaid) according to the manufacturer's instruction. cDNA synthesis was performed using Reverse Transcription Kit II Smobio according to the manufacturer's instruction. Mix A consisted of 3 µL RNA, 1 µL oligo (dT), and 1 µL DEPC-H_2O. Mix B consisted of 2 µL 5× RT buffer (DTT/dNTPs), 2.5 µL DEPC-H_2O, and 0.5 µL Rtase/Enzyme mix. PCR cycling conditions were followed: mix A was preheated at 70°C for 5 min and then incubated in ice for 1 min. Mix A and mix B were mixed, followed by DNA synthesis at 25°C for 10 min and 40°C for 50 min and final termination at 85°C for 5 min.

*fos*E gene expression using quantitative PCR (qPCR)

*fos*E gene expression was calculated using qPCR that consists of 5 µL SsoFast EvaGreen supermix, 1 µL primer forward, 1 µL primer reverse, 1 µL template cDNA, and 2 µL water-free DNase. qPCR cycling conditions were as follows: preliminary denaturation at 95°C for 30 s, followed by 39 cycles of denaturation at 95°C for 5 s, amplification at 59°C for 30 s and 65°C for 5 s, and one cycle of melt curve at 95°C for 5 s. All samples were measured in duplicate. Gene expression was measured using the $2^{-\Delta\Delta CT}$ method, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as the normalized standard.

RESULTS AND DISCUSSION

Growth curve of *L. casei* strains AP and AF in inulin

Lactobacillus casei strains AP and AF were grown in MRS first to produce biomass. After harvest, *L. casei* was inoculated in inulin. *L. casei* strains AP and AF were in a lag phase for 0 to 2 h as marked by a low level of OD and log phase for 2 to 12 h as shown by increasing OD from 0.1 to 2 for *L. casei* strain AP and 0.7 for *L. casei* strain AF (Figure 1). Stationary phase was marked as a stable OD value at 14 to 24 h incubation. *L. casei* strain AP grew twice faster in inulin than *L. casei* strain AF. This difference in the ability to grow in inulin showed that *L. casei* strains AP and AF have different metabolism systems to hydrolyze inulin. Previous studies reported that the inactivated *msm* gene of *L. acidophilus* made slow growth in FOS medium, whereas the inactivated *bfr*A gene made *L. acidophilus* not grow in FOS medium (Barrangou et al. 2003).

The growth rates of *L. casei* strain AP and AF in this study were faster than those of *L. casei* strain AP reported previously (Widodo et al. 2017). In the previous study, *L. casei* strain AP showed a lag phase at 0 to 6 h of incubation and a log phase at 6 to 12 h of incubation. This is because *L. casei* strains AP and AF have adapted for growth in inulin, so the lag phase was shorter.

Inulin and fructose determination in extracellular *L. casei* strains AP and AF

At 0 h of incubation, inulin was detected at 6,126.6 ppm, and fructose was detected at 571.65 ppm in extracellular *L. casei* strain AP. The level of inulin decreased quickly in the log phase at 0 to 12 h of incubation. Inulin was completely hydrolyzed at 20 h of incubation, indicating that *L. casei* strain AP entered a stationary phase. This result was similar to *L. plantarum* P14 and P17, which hydrolyze inulin completely in 24 h (Buntin et al. 2017). Fructose in *L. casei* strain AP was detected as hydrolyzed inulin's product from 571.6 to 51.6 ppm for 18 h (Figure 2). Fructose concentration decreased along with incubation. This is likely because fructose was transported to the cell. Fructose was not detected at 18 h of incubation and rose at 20 h of incubation to 2,026.35 ppm. If two or more sugars were added to the medium, *Lactobacillus* will metabolize simple sugars first (Buntin et al. 2017). Fructose will be transported to the cell and metabolized until there was no glucose anymore. After that, inulin will be degraded again, and fructose concentration will increase rapidly.

Inulin concentration in *L. casei* strain AF was 19,256.1 ppm in 0 h of incubation (Figure 3). This concentration was higher than that in extracellular *L. casei* strain AP. This shows that *L. casei* strain AP hydrolyzes inulin faster than *L. casei* strain AF. The difference was possibly because bacteria have some genes that encode sugar transporters such as PEP-dependent sugar PTS (Kleerebezem et al. 2003). Inulin concentration decreased during incubation. Inulin degradation was high in the log phase at 0 to 12 h from 19,256.1 to 5,194.7 ppm, and inulin concentration was still available at 22 h of incubation up to 4,892.6 ppm (Figure 3), although *L. casei* strain AF entered the stationary phase. This shows that *L. casei* strain AF cannot metabolize all inulin in media.

In *L. casei* strain AF, fructose was detected at 0 h of incubation and decreased during incubation from 1,655 to 536.6 ppm (Figure 3). This is likely because fructose was transported to the cell for catabolism. Fructose concentration in *L. casei* strain AF was higher than that in *L. casei* strain AP. A higher fructose concentration was a repressor for the *fos*E gene (Barrangou et al. 2006). This means that inulin cannot be hydrolyzed further until *L. casei* strain AF entered the late stationary phase; therefore, the OD of *L. casei* strain AF was lower than that of *L. casei* strain AP. FOS with DPs of 2 and 3 was faster to be metabolized than FOS with DP 4 in *L. paracasei* 1195. Transport and hydrolysis FOS was induced by sucrose and oligosaccharides with higher DP and suppressed by the hydrolysis product. However, a higher FOS concentration did not suppress fructose transport (Kaplan and Hutkins 2003).

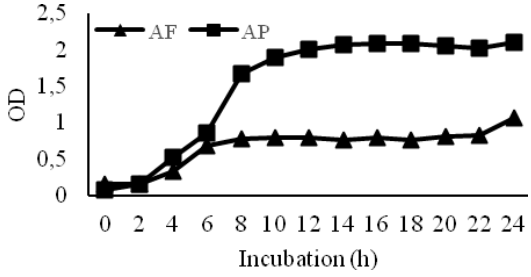

Figure 1. Growth curves of *Lactobacillus casei* strains AP and AF in inulin

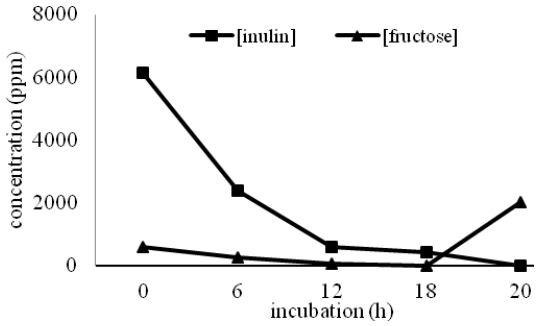

Figure 2. Inulin and fructose concentration in extracellular *Lactobacillus casei* strain AP

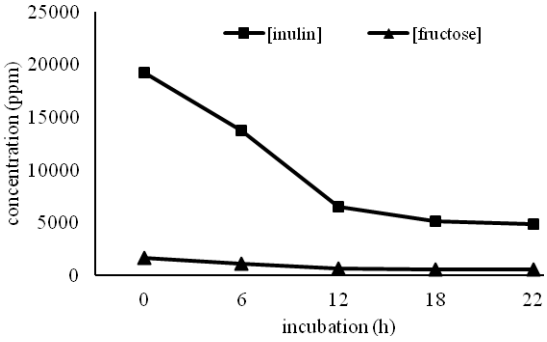

Figure 3. Inulin and fructose concentrations in extracellular *Lactobacillus casei* strain AF

Inulin and fructose determination in intracellular *L. casei* strains AP and AF

Inulin was detected at 2 h of incubation in intracellular *L. casei* strain AP and decreased after 6 h of incubation from 10,433.19 to 9,730.35 ppm. After that, inulin rose again at 12 h of incubation and became 10,739.46 ppm (Figure 4). This is because extracellular fructose concentration was low (51.6 ppm; Figure 2). FOS induced expression of *fos* operon in *L. casei* strain AP. Inulin concentration was also detected in intracellular *L. casei* strain AF from 9,766.06 ppm and decreased until 5,826.46 ppm (Figure 5). Intracellular inulin concentration was higher than extracellular inulin because of some β-

fructosidase anchored in cell walls like in *L. paracasei* (Velikova et al. 2017) and some β-fructosidase bacteria found intracellularly like in *L. plantarum* WCF1 (Kleerebezem et al. 2003). In this study, we did not isolate the cell wall of *Lactobacillus*, so perhaps, the β-fructosidase enzyme accumulated in the cell.

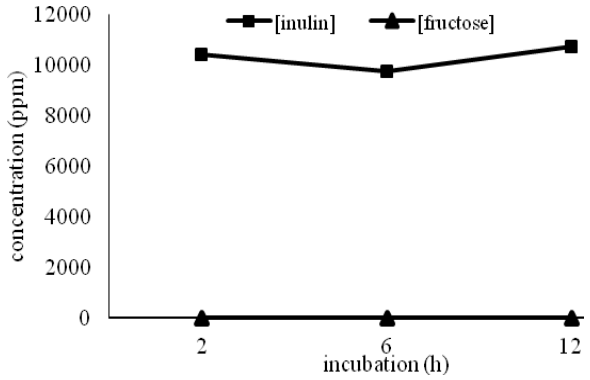

Figure 4. Inulin and fructose concentration in intracellular *Lactobacillus casei* strain AP

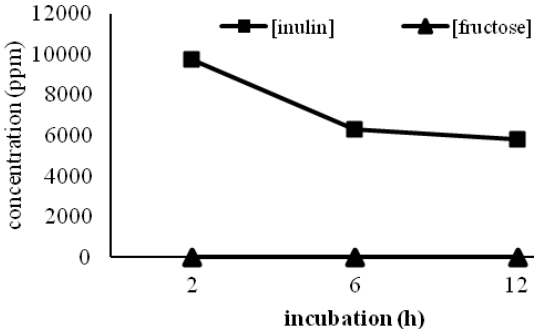

Figure 5. Inulin and fructose concentration in intracellular *Lactobacillus casei* strain AF

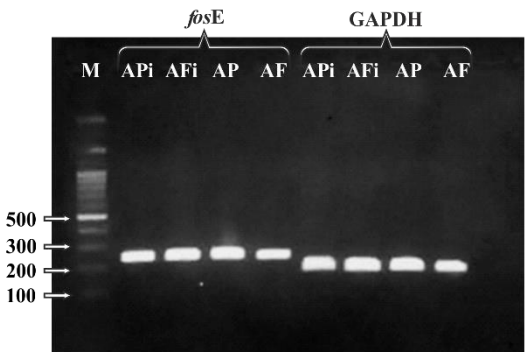

Figure 6. Amplification of *fos*E gene and GAPDH at the DNA of *Lactobacillus casei* strain AP and AF in MRS (AP and AF) and inulin (APi and AFi; M = marker DNA)

Fructose concentration was not detected in *L. casei* strains AP and AF. This is because fructose was in linkage with phosphate when it transported to the cell and entered the glycolysis pathway to generate ATP (Buntin et al. 2017). HPLC results showed another peak in time retention at 3.646 min, indicating inulin hydrolysis to another sugar, perhaps short-chain inulin. This indicated that the enzyme was acting as exo- and endoinulinase. Exoinulinase hydrolyzes inulin at terminals producing monosaccharides, whereas endoinulinase acts at residue nonterminal producing short-chain inulin (Velikova et al. 2017). Mn^{2+} concentration also plays a role in the catalytic activity of β-fructosidase. In low Mn^{2+}, β-fructosidase degrade inulin at random location (Petrov et al. 2017). This makes inulin to be hydrolyzed in varied products such as fructose, disaccharides, and short-chain inulin.

*fos*E gene amplification in *Lactobacillus casei* strains AP and AF

*fos*E gene in *L. casei* strains AP and AF was detected in MRS and inulin media. We used *fos*E primer pairs, which was designed using *L. casei* strain AP amplicon that detects a β -fructosidase gene fragments with a length of 501 bp (Aditiyarini 2016). We designed a primer that amplifies a short sequence. This is because we wanted to know an expression using qPCR that needs a short cDNA amplicon ranging from 100 to 300 bp. The amplicon product of *fos*E gene suspect was 236 bp. The *fos*E gene was detected in *L. casei* strains AP and AF at MRS and inulin media (Figure 6). *L. casei* strains AP and AF have *fos*E genes shown by the DNA band between 200 and 300 bp. The presence of the *fos*E gene proved that β -fructosidase degrades inulin, so the concentration was decreased, and fructose was detected. In this study, we used the GAPDH gene as an internal control. This enzyme catalyzes the sixth step of glycolysis for generating ATP. The *fos*E gene in *L. plantarum* P14 is 2,900 bp, and that in *L. plantarum* P76 is 2,386 bp (Buntin et al. 2017).

Sequence identification of *fos*E gene

The DNA amplicon was identified to determine the *fos*E gene length sequence. The *fos*E gene length was shorter than our prediction. The length of the *fos*E gene fragment in *L. casei* strains AP and AF was 229 bp. This sequence was used to align with other genes for sugar metabolism in the National Center for Biotechnology Information database. We used the fructan gene cluster in *L. paracasei* strain W20 (accession number MH047828.1), β-fructosidase (*fos*E) gene in *L. paracasei* strain LC1 (KU666517.1), sugar multi-transport region in *L. paracasei* strain LPC-S01 (LN846901.1), fructan β-fructosidase (*inu*) gene in *L. paracasei* subsp. *paracasei* strain DSM 23505 (KP663715.1), *fos* gene cluster in *L. paracasei* strain 1195 (DQ396803.1), and *lev*HX1 and *lev*H1 genes in *L. casei* (AB185852.1). All of these sequences share 99.03% identity. From the phylogenetic tree, all databases shared a common ancestor with *L. casei* strain AP, whereas *L. casei* strain AF did not (Figure 7). In this study, primer pairs for *fos*E genes amplification in *L. casei* strain AP was designed from the inulinase gene sequences from *lactobacillus* sp.

(Aditiyarini 2016). *Inu* gene from *L. paracasei* subsp. *paracasei* strain DSM 23505 and *L. casei* strain AP, *fos*E gene from *L. paracasei* strain 1195, and *L. paracasei* encode β -fructosidase by hydrolyzing inulin extracellularly. The *fos*E gene encodes β -fructosidase anchored in the cell membrane (Velikova, et al, 2017). *Lev*H1 encodes protein in the cell surface. LevH1 hydrolyzes inulin in an exo-type mode, so the product was fructose only and transported it to the cell via the PTS system (Kuzuwa et al. 2012).

*fos*E gene expression in *L. casei* strains AP and AF induced by inulin

*fos*E expression was induced by inulin or oligosaccharide. We grew *L. casei* strains AP and AF in inulin medium to induce *fos*E gene expression. We used *L. casei* strains AP and AF to grow in MRS as control. *fos*E gene expression in *L. casei* strain AP was up-regulated 5.9-fold, whereas *L. casei* strain AF was up-regulated 1.7-fold (Figure 8). The higher *fos*E gene expression in *L. casei* strain AP was concomitant with the ability of bacteria to degrade inulin better than *L. casei* strain AF. This was proven by the low inulin concentration in extracellular *L. casei*. *L. plantarum* strains P76 and P14 of *fos*E gene were up-regulated 10- and 25-fold when induced by inulin (Buntin et al. 2017).

One of the medium compositions of *L. casei* strains AP and AF is $MnSO_4$. Mn^{2+} increases the activity of β-fructosidase, thus increasing inulin degradation. Mn^{2+} also acts to help autophosphorylation at enzyme I. This enzyme is involved in the transport of fructose through the PTS system so that fructose can be transported to the cell (Petrov et al. 2017). A *DeoR* family transcriptional regulator was detected in *L. casei* strain AP (Widodo et al. 2017). This protein acts as a regulator in the catabolism system. This gene was regulated in transcription level by carbon catabolite repressor (ccr). This occurs when two or more sugars are added in the medium. High glucose concentration will bind with histidine protein kinase (HPrK). When HPrK is phosphorylated, it binds with catabolite control protein A, becomes complex, and attaches with a catabolite responsive element in the upstream. This complex makes transcription not to proceed so the *fos*E gene becomes down-regulated (Petrov et al. 2017).

In conclusion, *L. casei* strain AP was able to hydrolyze inulin better than *L. casei* strain AF as indicated by the low level of inulin in cells compared to *L. casei* strain AF. *fos*E gene was detected as 229 bp in *L. casei* strains AP and AF. *fos*E gene expression had an increase of 5.9 times in *L. casei* strains AP in the inulin medium and 1.7 times in *L. casei* strain AF compared to the MRS medium. *fos*E gene expression in *L. casei* strain AP was three times that of *L. casei* strain AF, so inulin was better metabolized by *L. casei* strain AP.

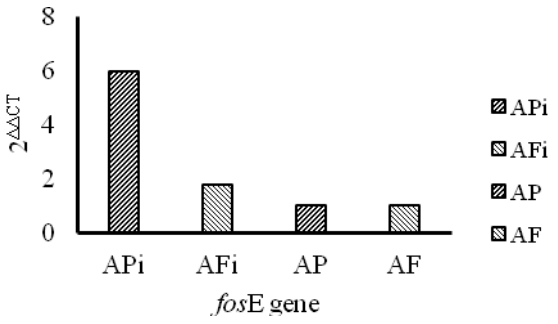

Figure 8. *fos*E gene expression in *Lactobacillus casei* strains AP and AF

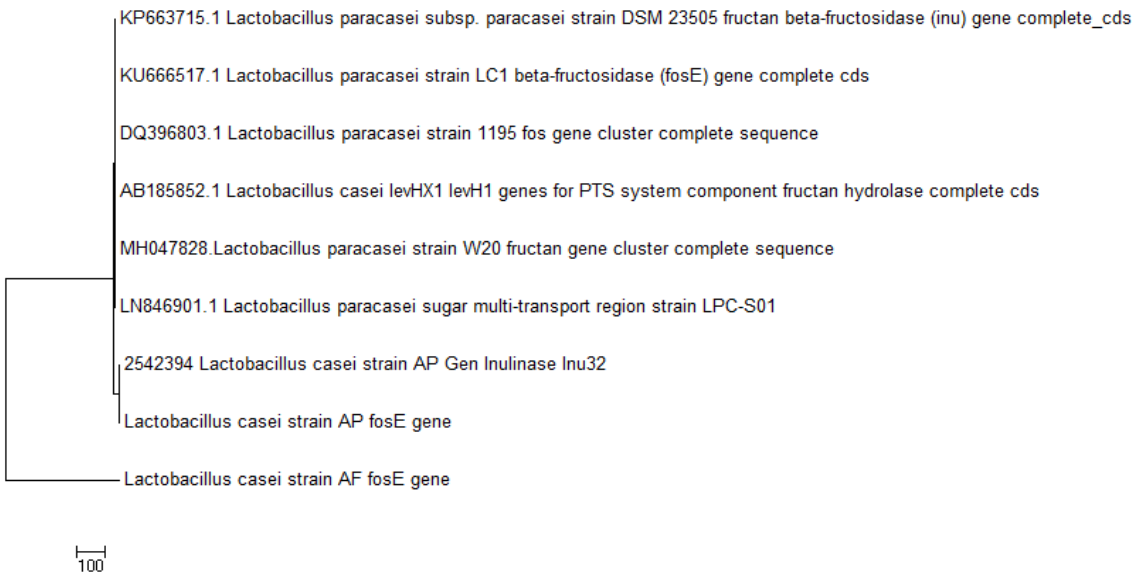

Figure 7. Phylogenetic tree of sugar metabolism in *Lactobacillus*

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Authors' contributions: ACA, TDW, and WD designed the study. ACA carried out the laboratory work. ACA, TDW, and WD analyzed the data and wrote the manuscript.

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