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Komagataeibacter xylinus as a novel probiotic candidate with high glucose conversion rate properties

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Abstract

Promoting general health in terms of obesity and diabetes prevention is recommended by health care systems. The objectives of this study were to isolate an efficient glucose-converting *Komagataeibacter xylinus* to cellulose and to evaluate the safety of the selected strain as a new generation of probiotics in the fight against obesity. Of the 97 samples, 43 *K. xylinus* strains were isolated and evaluated for their glucose conversion rate and 5 strains were examined for probiotic activities by *in vitro* assays. A strain with significant performance was fed to rats in order to determine its safety status *in vivo*. The results revealed that the strain K.X.1 had high level of glucose conversion rate and significant survival rate in acidic pH and bile salt. No adverse clinical signs and bacterial translocation to rats' organs were observed. The results showed that the strain of *K. xylinus* K.X.1 has suitable probiotic properties.

Keywords: Food science, Natural product chemistry, Microbiology, Food technology, Nutrition

1. Introduction

Promoting general health in terms of obesity and diabetes prevention is a matter of global concern for public health (Ali et al., 2010; Ma and Mu, 2016). One of the approaches recommended by health care systems is the consumption of probiotics which have therapeutic benefits on human (Moura et al., 2016; Lollo et al., 2015; Chiquette, 2009; Bassaganya-riera et al., 2012). Hill et al. defined probiotics as: “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (Hill et al., 2014). *Lactobacillus* and *Bifidobacterium* are the most famous probiotics which have been supported by many studies as providing potential benefits (Batista et al., 2015). Therefore, scientific evidence has increased rapidly to develop probiotic products such as fermented milks, yogurt and ice creams (Silva et al., 2018; Balthazar et al., 2017). Since the valuable effects of probiotics are different from strain to strain, some studies have been conducted to isolate and identify new strains that might be of help to prevent specific diseases in combination with other methods (Sahadeva et al., 2011).

From different published studies, kombucha tea and traditional apple vinegar have been considered to be one of the natural sources for weight control and diabetes treatment with unclear mechanisms (Petsiou et al., 2014; Mitrou et al., 2010a,b). This might have been due to the fact that microorganisms are responsible for these effects. One of the microorganisms in kombucha tea making process is *K. xylinus* which is also found in vinegars (Goh et al., 2012; Stasiak and Blazejak, 2009). The bacterium *K. xylinus*, a member of acetic acid bacteria, is a Gram-negative, aerobic, and nonpathogenic bacterium that produces cellulose by absorbing sugars like glucose and fructose (Garrity et al., 2004; Rangaswamy et al., 2015; Gullo and Giudici, 2008). Therefore, consuming the bacteria may help to reduce glucose absorption and prevent weight gain by converting glucose into cellulose in the human gut.

To the best of our knowledge, no studies have been carried out to determine glucose conversion rate and to characterize probiotic properties for *K. xylinus* so as to determine its effectiveness in decreasing intestinal glucose absorption. *In vitro* selection and characterization criteria for potential probiotic strains are acid and bile resistance activities which allow the microorganisms to stay alive in the gastrointestinal tract. This study was conducted to isolate the strain with high glucose conversion rate from traditional vinegar and its ability to survive in the condition of gut tract like resistance in acid, bile, low oxygen pressure, and high temperature in order to beneficially affect human health by modulating glucose metabolism to help people suffering from obesity and diabetes. The pathogenicity of the selected strain was evaluated in large amount of consumption in Wistar rats.

2. Materials and methods

2.1. Sample collection and identification of *K. xylinus* strain

Samples of traditional apple vinegars were collected. Each sample was separately packed in bags and was transported to the laboratory. Strains of *K. xylinus* were selected using the following procedure: 1 ml of each sample was plated on GYC medium (5% glucose, 1% yeast extract, 3% calcium carbonate, and 2% agar). All chemicals were purchased from Sigma-Aldrich. Colonies with clear zone were cultured on solid Hestrin-Schramm (HS) medium (2.0% D-glucose, 0.5% peptone, 0.5% yeast extract, 0.27% Na₂HPO₄, 1.15% citric acid, and 1.5% agar). Mucosal colonies which appeared on the medium were identified with standard phenotypic and molecular analyses. All experiments were performed at 37 °C in order to isolate strains that withstand high temperature as compared to its own niche (18–22 °C). Table 1 shows the summary of the characterization tests (Suwanposri et al., 2013; Garrity et al., 2004; Aydin and Aksoy, 2009). The reference strain was *Gluconacetobacter xylinus*; PTCC No.: 1734. A polymerase chain reaction (PCR)-based procedure was used to isolate original ribosomal DNA. PCR primers included the forward primer (27 F; '5-AGAGTTTGATCMTGGCTCAG-'3) and the reverse primer (1492 R; '5'-CGGTTACCTTGTTACGACTT-'3). PCR was carried out in a mixture including DNA template 1 µl, 1 µl of each primer, and 12 µl of Taq DNA Polymerase Master Mix RED (Ampliqon), adjusted to a total volume of 25 µl with sterile deionized water. Thermal cycling condition included initial denaturation at 95 °C for 2 min,

Table 1. Bacteriological identification tests used for 43 strains of *K. xylinus* isolated from traditional apple vinegar.

Characterization tests	<i>K. xylinus</i>
Catalase	+
Oxidase	-
Indole production	-
Sodium citrate utilization	-
Methyl red	-
Voges-Proskauer	-
H ₂ S formation	-
Urea utilization	-
Cellulose production	+
Growth on 3% (v/v) ethanol in the presence of acetic acid 5–8%	-
Gelatin liquefaction	-
Requirement of acetic acid for growth	-
Growth on malachite-green 0.01% agar	-

followed by 30 cycles of template denaturation at 95 °C for 30 s. The annealing and extension were performed at 56.2 °C for 30 s and at 72 °C for 150 s, respectively. The final cycle was followed by incubating the reaction mixture at 72 °C for 5 min. Chromosomal DNA was extracted using the Phenol-Chloroform isoamyl alcohol method. As subsequently shown, 600 µl of extraction buffer (1 M NaCl, 1 M Tris-HCl, and 0.05 M EDTA), 13 µl of sodium dodecyl sulfate (SDS 25%), and 3 µl proteinase K (20 mg/ml) were added to the washed pellet of each isolated bacteria. It was incubated for 1 h at 60 °C. Equal volume of phenol: chloroform: isoamyl alcohol solution was added to the mixture and it was centrifuged at 4 °C in 14000 rpm for 10 min. Aqueous phase (200 µl) was separated and 620 µl of chloroform was added. After centrifugation, DNA was precipitated using 2 volumes of isopropanol (-20 °C). The mixture was incubated for 2 h at -20 °C. DNA was washed using 75% ethanol and was later air dried. The quantity of the DNA dissolved in the double distilled water was measured using Nanodrop spectrophotometer (260 nm).

2.2. *In vitro* glucose conversion rate

The glucose conversion rate of each isolated strain to cellulose was measured using enzymatic assay of glucose oxidase (GO). Briefly, each isolated bacteria with the adjusted count (1×10^8 cfu/ml using photometric assay) was inoculated into 100 ml of HS broth medium. Samples were collected at the time of 0, 3, 6, 9, 24, 27, 30, 33, 36, and 48 h after incubation. The GO assay was performed according to the kit manufacturer's instruction (GOD-PAP, PARS AZMUN). Reference strain and *Escherichia coli* (PTCC No.: 1338) were used as positive and negative controls, respectively. Each isolate was tested trice.

2.3. Acid tolerance assay

Acid tolerances of the strains were determined following the procedure described subsequently. One millilitre of HS broth containing approximately 10^9 CFU/ml of the bacteria was transferred into 9 ml phosphate buffered saline (PBS) with adjusted pH of 2 and 4 and it was incubated at 37 °C for 3 h. Acid tolerance of each strain was evaluated by calculating the ratio of viable cell cultured on HS agar to surviving cells after incubation at pH 2 and 4 for 3 h at 37 °C (Kazem et al., 2017; Ragul et al., 2017).

2.4. Bile tolerance assay

Bile tolerance of each isolated strains was obtained through the following procedure: suspensions of isolated bacteria were cultured in nutrient broth medium containing

0.3% bile oxalate. The medium was incubated at 37 °C for 8 h. Inhibition of growth was measured by monitoring the optical density at 600 nm. Medium without bile was used as blank. The ratio of viable cells reflecting coefficient of inhibition (Cinh) was calculated using the formula presented by [Argyri et al. \(2013\)](#). Based on Cinh, strains were divided into two groups: resistant to 0.3% bile salts ($0.2 < \text{Cinh} < 0.4$) and sensitive to it ($\text{Cinh} > 0.4$).

$$\text{Cinh} = \frac{\Delta\text{T8} - \text{T0 (control)} - \Delta\text{T8} - \text{T0 (treatment)}}{\Delta\text{T8} - \text{T0 (control)}}$$

where T0: absorbance reading at 0 h and T8: absorbance reading at 8 h.

2.5. Isolation of strain able to grow in low oxygen pressure

To obtain a pure culture with the ability to grow in a lower oxygen environment, isolated strains were allowed following the conditions that follows. Each isolated strain was cultured in the HS medium and incubated at 37 °C in a microaerophilic candle jar (5% O₂, 10% CO₂, and 85% N₂). Colonies that were able to grow and produce cellulose in the mentioned condition were stored for animal study.

2.6. Animals and experimental design

Two groups of six male Wistar rats (*Rattus norvegicus*) were sacrificed to evaluate the pathogenicity of the bacteria. These rats were maintained at 22 °C, 50% relative humidity, and 12/12 h light/dark regimen. To evaluate the pathogenicity of the bacteria in large amount of consumption, 10¹⁶ CFU of the selected bacteria were fed to the rats. Six rats were fed with 10¹⁶ CFU of bacteria, while the other group was given distilled water. The trial lasted for a month. Animal study was conducted under welfare and ethics guidelines approved by Tehran University of Medical Science. General health of the rats was monitored daily as earlier described [Shu et al. 1999 \(Table 2\)](#). Daily food intake and weekly body weight were recorded during the study. Occurrence of diarrhea and vomiting was monitored daily. Rats' livers, intestines, and spleens were removed after euthanasia for histopathological study.

2.7. Histopathological analysis

Bacterial translocation from the gut to distant organs was studied through histopathological examination. Three tissue sections of each rat's liver, intestine, and spleen were aseptically collected. They were washed in normal saline and fixed in formalin (10%) for 48 h. Dehydration was performed using alcohol. Samples were cleared in xylene and stained with hematoxylin and eosin ([Azerêdo, 2010](#)). These samples were observed by examination at least 10 zones of each tissue section under light microscopy.

Table 2. General health appearance of rats was daily monitored using a score system described by Shu et al. 1999

General health appearance	Score
Mouse bright-eyed alert, has a smooth coat with a sheen, responds to stimulus, shows interest in its environment	1
Fur slightly ruffled, a loss of sheen to the coat, mouse remains alert and active	2
Fur noticeably ruffled, parts of coat form clumps, mouse not as alert or active, less interested in environment outside of cage, signs of hyperventilating when handled	3
Mouse hunched over and sleepy, little interest shown in environment, fur clumped	4
Mouse non-reactive to stimulus, fur has a “bottle brush” appearance, <i>i.e.</i> , standing on end, mouse hunched over preferring to sleep rather than react to environment, mouse cold to touch, paws are cold to touch	5

The study protocols were reviewed and approved by Research Ethics Committee of Tehran University of Medical Science.

3. Results

3.1. Identification of *K. xylinus* strains

According to standard bacteriological and physiological analyses, 43 strains of *K. xylinus* were isolated from 97 samples of traditional vinegars. Full sequence of 16S rDNA gene of each isolate was compared with the sequences deposited on the NCBI. Following BLAST analysis, the 16S rDNA gene sequence showed that the strain had 99% identity to *K. xylinus*. The 16S rDNA gene sequence determined in this study has been published in Genbank under accession number KY711526.

3.2. *In vitro* glucose conversion rate

Glucose conversion rate of each isolate to cellulose was determined using GO kit by measuring the reduction of the glucose concentration in the medium. Among the 43 strains, 5 have the ability to convert glucose to cellulose in the HS medium in 48 h of inoculation at the highest level. Following the results shown in Fig. 1, the strain K.X.1 showed the highest glucose conversion rate by reflecting the reduction of the glucose concentration in the medium. Then, it was selected for further *in vitro* probiotic profile investigation.

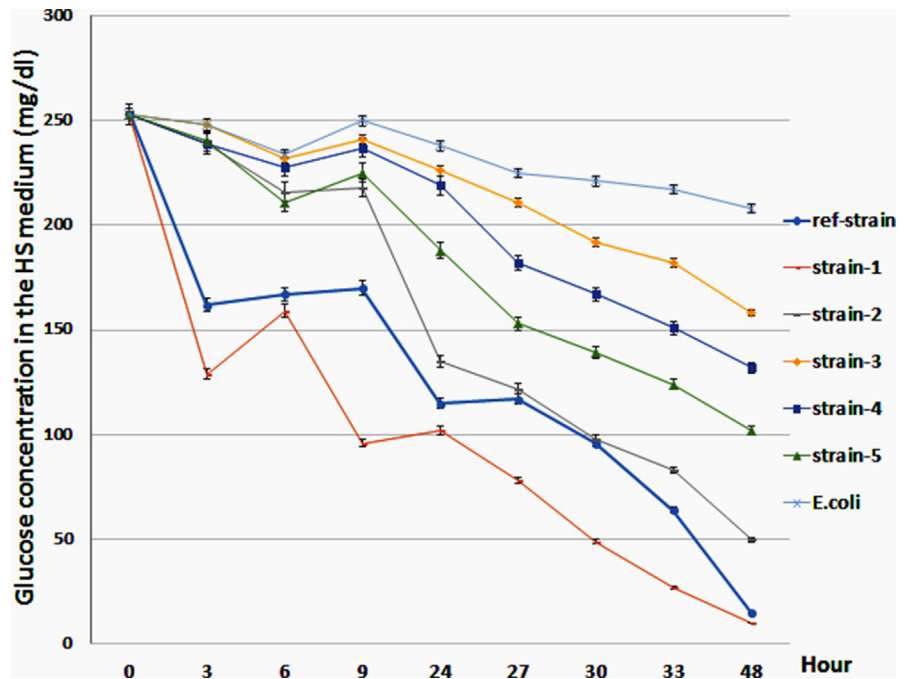


Fig. 1. Glucose conversion rate of five isolates (glucose concentration in the HS medium, mg/dl); reference strain (*K. xylinus*) and *E. coli* were tested as positive and negative control, respectively. Strain-1 (K.X.1) showed high level of glucose conversion rate. Each point in the plot represents the mean \pm SD of three independent experiments.

3.3. Survival rate of *K. xylinus* strains at different pH value and bile salt

Acid and bile tolerance of 5 *K. xylinus* strains was determined. The results showed that all the strains had significant survival rate to acidic environment. Strains K.X.1, K.X.2, K.X.3, and K.X.4 showed higher acid tolerance and K.X.5 showed lower acid tolerance. According to the colony count performed after 3 h at different pH value, no significant decreases were observed in the loss of viability of the 4 isolated strains.

Also, strains K.X.1, K.X.2, K.X.4, and K.X.5 showed higher resistance to the presence of 0.3% oxgall bile after 8 h. The coefficient of inhibition of the tested bacteria was between 0.2 and 0.4 ($0.2 < C_{inh} < 0.4$). Strain K.X.3 was the most sensitive strain among the other isolated bacteria. The results are shown in Table 3.

3.4. Isolation of strains able to grow in low oxygen pressure

From the tests conducted in this study, strains K.X.1, K.X.2, and K.X.4 were qualified to be as a probiotic candidate. Among these three strains, K.X.1 and K.X.2 have

Table 3. Results of acid and bile salt tolerance of five selected *K. xylinus* strains and reference strain.

Strain	Acid tolerance						Bile tolerance (0.3%) C _{inh}
	pH 7 (control)		pH 4 (treatment)		pH 2 (treatment)		
	hour						
	0	3	0	3	0	3	8
ref	5.1×10 ⁹	5.6×10 ⁹	5×10 ⁹	4.2×10 ⁹	5.8×10 ⁹	5.1×10 ⁸	0.35
K.X.1	3.8×10 ⁹	4.2×10 ⁹	3.2×10 ⁹	3×10 ⁹	4.1×10 ⁹	3.8×10 ⁸	0.21
K.X.2	1.5×10 ⁹	1.7×10 ⁹	1.4×10 ⁹	1.1×10 ⁸	1.9×10 ⁹	1.1×10 ⁷	0.17
K.X.3	7.3×10 ⁹	7.8×10 ⁹	6.9×10 ⁹	5.3×10 ⁹	7.2×10 ⁹	6.4×10 ⁷	0.45
K.X.4	8.3×10 ⁹	8.6×10 ⁹	8.5×10 ⁹	8.2×10 ⁸	8.4×10 ⁹	7.3×10 ⁸	0.26
K.X.5	2.1×10 ⁹	2.5×10 ⁹	3.9×10 ⁹	3.6×10 ⁸	3.1×10 ⁹	2.4×10 ⁶	0.40

Each value represents mean ± SD from three trials (log CFU/ml).

the ability to grow and produce cellulose in low oxygen pressure. Considering the results of glucose conversion rate and *in vitro* probiotic profiles, K.X.1 was finally selected for animal study.

3.5. Safety assessment

To test the pathogenicity and cytotoxicity of the selected strain, it was fed to rats. No adverse effects were observed on the general health of rats fed with high dose of bacteria as compared to the control rats. No considerable differences were recorded in feed intake, water consumption, and weight gain between the control group and the fed rats group. Bacterial translocation to the organs was also evaluated. There

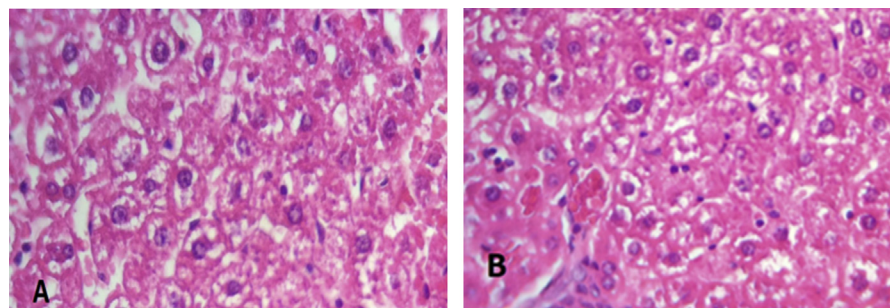


Fig. 2. Histopathology of rats' liver; A) Liver of rat treated with K.X.1, B) Liver of rat given distilled water (control). Bacterial translocation was not observed in the organ. Cells appearance and red cytoplasm were observed to be similar to that of the rats given distilled water.

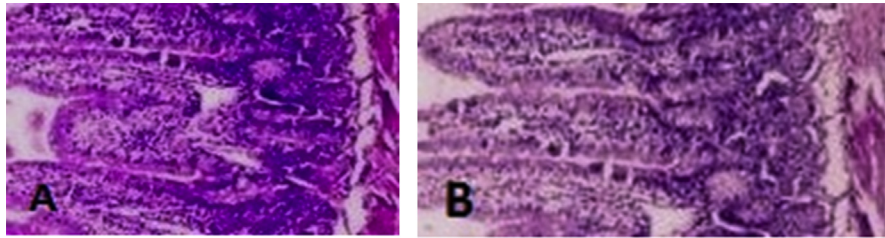


Fig. 3. Histological section of rats' gut; A) Intestine of rat fed with K.X.1, B) Intestine of control rat. No bacterial translocation and histopathological signs were detected.

was no translocation of bacteria in the tissue samples. There were no signs of inflammation, degeneration, and necrosis in the organs' cells (Figs. 2, 3, and 4).

4. Discussion

Of the 43 isolates, 5 strains of *K. xylinus* with significant glucose conversion rate were selected from traditional apple vinegars. In order to introduce a potential probiotic strain to treat obesity and diabetes, probiotics properties of each isolate were assessed *in vitro* by modulating glucose absorption in conjunction with other routine methods.

It has been reported in several published surveys that consumption of vinegars especially apple vinegar and kombucha tea has significant effects on reducing blood sugar and losing weight. However, these mechanisms remain unclear (Petsiou et al., 2014; Mitrou et al., 2010a,b; de Dios Lozano, 2012). The microbiological composition of kombucha tea and apple vinegars showed that *K. xylinus* is the main microorganism in them (Bellassoued et al., 2015). It might be that the

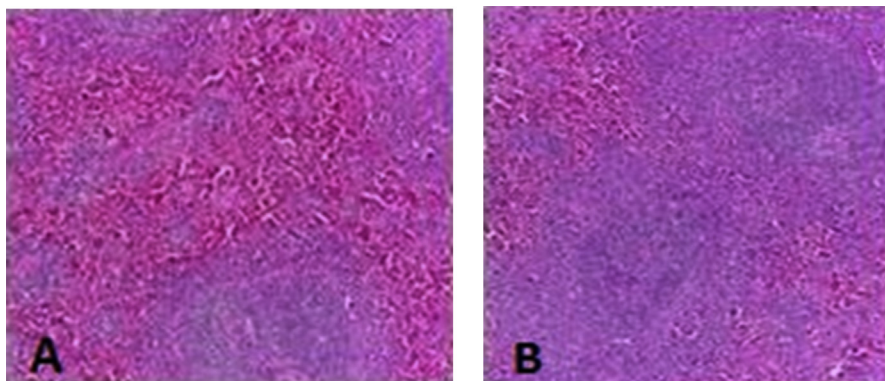


Fig. 4. Histopathology of rats' spleen; A) Spleen of rat fed with K.X.1, B) Spleen of control rat. There is no bacterial translocation, inflammation and degeneration in cells.

bacterium is responsible for their beneficial effects as it has the ability to convert sugars to cellulose.

In the present study, glucose conversion rate was determined in 48 h using glucose oxidase method which is highly sensitive as compared to using Calcofluor-white staining and NaOH treatment, conventionally used for cellulose production assessment (Shpigel et al., 1998; Skinner and Cannon, 2000; Çoban and Biyik, 2011). The cellulose production rate is directly associated with glucose reduction rate in the medium (Scott Scott Williams and Cannon, 1989). It also reflects glucose absorption activity by the strains of *K. xylinus* (Santos et al., 2013; Kurosumi et al., 2009; Czaja et al., 2004; Saxena et al., 2004). The results revealed that the glucose reduction rate is different between strains. Therefore, 5 strains which showed high level of glucose absorption were selected and the probiotic activities for each of the strains were assessed.

No studies have been carried out to evaluate the probiotic properties for *K. xylinus*. In this study, probiotic properties were evaluated for the strains which showed high glucose absorption. From previous surveys, one of the most important criteria for selecting a microorganism as a probiotic is its ability to stay alive under gastric environment such as low oxygen pressure and the presence of bile salts and acid (Saarela et al., 2000; Sahadeva et al., 2011; Ding et al., 2017; Bae et al., 2002).

All experiments were performed at 37 °C to imitate the optimal physiological temperature for human body. However, *K. xylinus* ideal growth conditions are at 18–22 °C and in aerobic environment (Chawla et al., 2009). K.X.1 strain which has the ability to grow and produce cellulose at 37 °C and lower oxygen condition was selected. Besides, the strain showed high level of glucose conversion rate. The ability of the bacterium to convert glucose to cellulose makes it suitable for using it in commercial probiotic products.

From the results of the present study, K.X.1 is recognized as a safe microorganism for human consumption. Safety status of K.X.1 was determined by feeding rats with 10^{16} doses of the bacterium. According to the global standards, approximately 10^6 to 10^8 of viable cells are required per millilitre to gain therapeutic benefits (Ozyurt and Ötles, 2014; Shiby and Mishra, 2013; Vijaya Kumar et al., 2016). Although rats were fed with large amount of bacteria, no clinical signs were observed. Histopathological experiments confirmed the findings. No presence of bacteria was observed in the organs. Histopathological studies also showed no presence of inflammation, degeneration or necrosis signs in the cells. Bacterial translocation to organs such as liver is the most useful indicator sign of pathogenicity of the bacteria. Moreover, to the best of our knowledge, there are no reports that Acetobacteraceae family

causes infection in human. They do not produce any toxins and virulence factors (Edberg, 1992).

Rats' blood glucose cannot be measured during the bacteria consumption as there are great amounts of various cellulolytic bacteria in their intestine (Macy et al., 1982; Queipo-ortun et al., 2013). On the other hand, glucose which had been converted to cellulose by K.X.1 in rats' gut can be reconverted to a former state by the cellulolytic bacteria. Therefore, decreasing intestinal glucose absorption cannot be detected. Also some factors related to glucose such as the weight loss and lipid profile of rats before and after administration of *K. xylinus* cannot be measured. Further investigation will be carried out in human to evaluate intestinal glucose absorption.

The bacteria adhering to intestinal epithelial cells were not assessed because there is no need to colonize the human intestine with the bacterium. On the other hand, these bacteria can be consumed regularly as planktonic probiotic in order to beneficially affect human health.

Conclusively, the results of this study showed that K.X.1 from traditional apple vinegar has high level of glucose conversion rate in 48 h and significant probiotic properties *in vitro*. Thus, the strain of the bacterium shows potential for use as a probiotic. Further research is needed to conclude that it can be consumed as a supplementary therapeutic probiotic in combination with other methods to promote human health for obesity and diabetes prevention by decreasing intestinal glucose absorption.

Declarations

Author contribution statement

Paria Sadat Lavasani, Elahe Motevaseli, Nafiseh Sadat Sanikhani, Mohammad Hossein Modarressi: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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