



Original article

Pharmaceutical significance of *Leuconostoc mesenteroides* KS-TN11 isolated from Nile Tilapia, *Oreochromis niloticus*Bilal Ahmad Paray^{a,*}, Irfan A. Rather^{b,1}, Mohammad K. Al-Sadoon^a, Al-Shammari Fanar Hamad^b^a Zoology Department, College of Science, King Saud University, PO Box 2455, Riyadh 11451, Saudi Arabia^b Department of Applied Microbiology and Biotechnology, Yeungnam University, Gyeongsan, Gyeongbuk 712-749, South Korea

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ABSTRACT

Aquatic animals are known for their myriad of beneficial bacteria with diverse biologically active compounds. The current study was aimed to isolate and characterize potentially beneficial lactic acid bacteria from Nile Tilapia and evaluate their pharmaceutical applications. The fish samples were dissected and stomach, intestine, and gills were collected and serially diluted for the isolation of lactic acid bacteria (LAB) on BCP agar media. Identification of isolate was carried by biochemical and molecular characterization using API kit and 16S rRNA gene sequencing analysis, respectively. Further, KS-TN11 was assessed for α -glucosidase inhibitory potential using the chromogenic method. A lactic acid bacterium KS-TN11 was isolated from the stomach of Nile Tilapia and identified as *Leuconostoc mesenteroides*. Effect of KS-TN11 on lipid accumulation in adipocytes was done by using Oil Red O staining. The isolate showed strong antibacterial activity against a number of pathogenic bacteria in vitro. In addition, *L. mesenteroides* KS-TN11 (50 mg/ml and 100 mg/ml) tends to inhibit adipogenesis in 3T3-L1 adipocytes and thus may have possible anti-obesity effects. Moreover, *L. mesenteroides* KS-TN11 exhibited substantial α -glucosidase inhibitory activities by 41.33% at 50 mg/ml and 64% at 100 mg/ml, respectively. The bacterium showed potent antibacterial activity against a number of pathogenic bacteria; in addition to alpha-glucosidase activity, and inhibition of lipid accumulation in 3T3-L1 cell line. These results reinforce KS-TN11 as a novel bacterium with an impending pharmaceutical application.

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1. Introduction

There are different types of indigenous lactic acid bacteria (LAB) present in nature, and aquatic animals hold a plethora of potential LAB with probiotic properties (Balcazar et al., 2008). Currently, LAB are used as probiotics adhering number of beneficial properties such as antibacterial activity (Rather et al., 2013), antifungal activity (Rather et al., 2014), immune modulation (Kamiya et al., 2017; Kim et al., 2012; Kober and Bowe, 2015; Tsai et al., 2010), anti-obesity (Tsai et al., 2014), and anti-diabetic properties (Bajpai et al., 2016) and so on. Due to the production of metabolic

characteristics, LAB are considered as novel bacteria for their applications in food and pharmaceutical industry.

Further, obesity has become a very common and serious lifestyle disease over past few decades and continues to increase in developing countries. However, obesity is preventable by choosing a healthier diet and lifestyle. Lactic acid bacteria have been used since ages in fermented food products and have a number of beneficial properties. There are vast number of reports suggesting an antimicrobial effect of *Lactobacillus* spp. against intestinal pathogens particularly against *Escherichia coli* O157:H7 and *Salmonella* spp. (Tsai et al., 2014; Bajpai et al., 2016). In addition, LAB also showed anti-obesity effect (Choi et al., 2007; Kim et al., 2008).

Diabetes is one of the loathsome chronic diseases among humans, especially in elderly people. Its dramatic increase worldwide has led to the increasing appearance of diabetes-related comorbidities. The disease has affected around 100 million elderly people aged 60–78 years old in 2010 and is expected to double in next 20 years (Shaw et al., 2010). There are a number of risk factors associated with diabetes such as food, genetics, or environment. Since diabetes is directly associated with the increase in blood glucose level, a number of reports suggesting that intake of LAB

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decreases the blood glucose level (Honda et al., 2012a, 2012b; Yun et al., 2009; Matsumoto et al., 2016). Several LAB are reported to decrease postprandial blood glucose level by suppressing the glucose absorption and decreasing the glucose available from digestion of foodstuff (Tabuchi et al., 2004; Honda et al., 2011; Honda et al., 2012a, 2012b). Nevertheless, insufficient studies have been done with other LAB species. In addition, in most of the reports, the effect of live bacteria has been studied (Honda et al., 2012a, 2012b).

In this study, the antibacterial, anti-obesity, and alpha-glucosidase potential of a fish isolate *L. mesenteroides* KS-TN1 were evaluated to confirm its pharmacological significance.

2. Materials and methods

2.1. Media and reagents

The de Man, Rogosa and Sharpe (MRS) agar medium were purchased from Difco (USA). The agar medium, Bromocresol Purple (BCP), p-nitrophenyl- α -D-glucopyranoside, yeast α -glucosidase, and 3,4-dihydroxy-L-phenylalanine (DOPA) were purchased from Sigma-Aldrich (Sigma, MO, USA). All other chemicals or reagents used were of analytical grade.

2.2. Target pathogenic strains

The highly pathogenic bacteria such as *Escherichia coli* O157:H7, *Salmonella enterica* ATCC 4731, *Listeria monocytogenes* KCTC 3569, *Staphylococcus aureus* KCTC 1621 and *Bacillus subtilis* KCTC 1021 were used as target bacteria. The strains were collected from American Type Culture Collection and Korean Type Culture Collection and were cultured on nutrient broth and agar at 37 °C. The stock culture samples were stored at –80 °C in cryopreservative vials.

2.3. Fish collection and isolation of LAB

Nile Tilapia, *Oreochromis niloticus* samples were captured using the net (weighed between 150 and 300 g) from the Wadi Namar in the western area of Riyadh, Saudi Arabia. The isolation of lactic acid bacteria was carried by sacrificing fish and dissect its gills, stomach, and intestine. The collected samples were homogenized for a short period of time and serially diluted using phosphate buffer saline (Cho et al., 2013). The dilutions were made from 10^{-1} to 10^{-7} and 100 μ l from each dilution was plated on BCP agar plates. The inoculated plates were incubated for 24 h at 37 °C. The clean zone around each colony was taken as lactic acid bacteria (Zapata, 2013). Further, the identified colonies were selected using inoculation loop and inoculated in the de Man, Rogosa and Sharpe (MRS) broth and incubated at 37 °C for 24 h. The samples were further spread on MRS plates and re-cultured for long-term preservation at –80 °C.

2.4. Antibacterial spectrum

The initial screening of fish isolates was carried out against pathogenic bacteria using agar well diffusion assay (Murray et al., 1995; Rather et al., 2013). Briefly, the plates of nutrient agar were prepared and allowed to solidify. The plates were spread with 200 μ l of target bacteria (10^7 cfu/ml) and allowed to dry for 10 min. The 24 h culture broths of 32 LAB isolates grown in MRS media at 37 °C were centrifuged at 10,000g for 10 min. The supernatant was collected and filter-sterilized through a 0.45- μ m-pore-size filter (Sartorius Stedim Biotech, Goettingen, Germany). An autoclaved borer was used to make uniform wells poured with

100 μ l filter sterilized cell-free-supernatant of the isolated bacterium. The plates were incubated for 24 h at 37 °C. After incubation, the antibacterial activity was determined by measuring the zones of inhibition against tested bacteria. The assay was done in triplicates.

2.5. Biochemical and morphological identification of fish isolate

Based on the initial screening of highly antibacterial activity against pathogenic strains, KS-TN11 was selected as a potential strain. The isolate was identified by observing the colony shape on BCP agar plates, and by Gram-staining and cell morphology under a microscope. Further, for biochemical characterization, API 50CH strips with API 50CHL medium was used as per manufacturer instructions (Shin et al., 2007; Rather et al., 2013). The strips were processed for analyzing the API profiles using computer API-LAB Plus Version.

The isolate was further characterized by 16S rRNA gene sequencing. The sequences were compared on National Center for Biotechnology Information (NCBI) for homology and submitted to Genbank.

2.6. Extraction and sample preparation

For extraction of biologically active metabolites, SK-TN11 was cultured in MRS broth for 24 h at 37 °C. The culture was mixed with ethanol (1:2 ratio v/v) and incubated on a rotatory shaker for 4 h at room temperature. Further, the mixture was centrifuged at 10,000 rpm for 15 min. The ethanol extract in the upper phase was collected and vacuum evaporated under reduced pressure at 40 °C. The sample was freeze-dried for three days and stored at 4 °C for further use.

2.7. Cell culture

The cell line 3T3-L1 (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) in presence of 10% fetal bovine serum in a humidified environment supplied with 5% CO₂ at 37 °C. The differentiation of 3T3-L1 preadipocytes to mature adipocytes were evaluated as reported previously (Tominaga et al., 2009). During treatments, unless otherwise stated, cells were maintained in complete medium containing KS-TN11 for 6–8 days before further analysis, and maturation medium was changed every 2 days. KS-TN11 was supplemented with both induction mediums. *Oil Red O staining*

On day 7, accumulation of lipid in adipocytes was evaluated by oil Red O staining. Briefly, after cell maturation, the control and KS-TN11 (50 mg and 100 mg) treated cells were washed with sterile 1X phosphate buffered saline (PBS), followed by one-hour fixation in 10% formalin at room temperature. Further, the cells were washed three times with sterile deionized water followed by coating with 0.6% Oil Red O dye in isopropanol and water (6:4 ratio v/v) for 20 min. Finally, the cells were washed 3–4 times with sterile deionized water before microscopic observation.

2.8. α -glucosidase inhibitory activity

LAB are known for its beneficial properties and have been used since ages for food fermentation. A large number of the food-based LAB are now potential probiotics. In this study, two concentrations of KS-TN11 were evaluated for its α -glucosidase inhibitory potential to confirm its anti-diabetic property using chromogenic method (Yuan et al., 2012; Bajpai et al., 2016). A 50 μ l of various concentrations (2.5 mg and 5 mg) of SK-TN11 and 100 μ l of α -glucosidase was dissolved in 0.1 M PBS, the solutions were gently mixed in a 96-well microplate and incubated for 10 min

at 25 °C. In addition, a 50 µl of p-nitrophenyl- α -D-glucopyranoside (5 mM) using PBS as a substrate solution was added after 10 min incubation to wells and the plate was again incubated for 5 min at 25 °C. However, the reading for absorbance was taken before and after incubation at 405 nm using microplate reader. Simultaneously, the absorbance of 50 µl PBS buffer was taken separately for comparison with test groups. The experiments were replicated

three times and α -glucosidase inhibitory activity was calculated using the formula:

$$\text{Inhibition (\%)} = \frac{(\text{Control absorbance} - \text{Sample absorbance})}{\text{Control absorbance}} \times 100$$

2.9. Statistical analysis

All data are expressed as the mean \pm SD., and comparison was made by using Student's *t*-test.

3. Results and discussion

3.1. Isolation and screening of LAB

The small colonies appeared yellow color on BCP agar confirms the presence LAB. In this study, 32 fish LAB were isolated from stomach, intestine, and gills of Nile Tilapia, *O. niloticus* as shown in Table 1. Further, each yellow colony was carefully picked and grown in MRS broth and checked for its antibacterial activity against various pathogenic bacteria.

3.2. Anti-bacterial activity and selection of LAB isolates from Nile Tilapia

The cell-free-supernatant of each isolate was checked for antibacterial activity against *Escherichia coli* O157:H7 and *Salmonella enterica* ATCC 4731, *Listeria monocytogenes* KCTC 3569, *Staphylococcus aureus* KCTC 1621 and *Bacillus subtilis* KCTC1021. The isolate KS-TN11 showed highest antibacterial activity (Fig. 1), and was chosen for further study.

3.3. Biochemical and molecular characterization of KS-TN11

The morphological identification of KS-TN11 was carried out according to Bergey's Manual of Systematic Bacteriology (Holt et al., 1994). The small colonies on BCP agar appearing in yellow color confirm the presence of LAB strain (Ashraf and Shah, 2011). The BCP is a specific media for the LAB as it inhibits the formation

Table 1
List of isolated lactic acid bacteria from Nile Tilapia (*Oreochromis niloticus*).

Lab name	Source	Media	Identification
KS-TN1	Stomach	BCP	Lactic acid bacteria
KS-TN2	Stomach	BCP	Lactic acid bacteria
KS-TN3	Stomach	BCP	Lactic acid bacteria
KS-TN4	Stomach	BCP	Lactic acid bacteria
KS-TN5	Stomach	BCP	Lactic acid bacteria
KS-TN6	Stomach	BCP	Lactic acid bacteria
KS-TN7	Stomach	BCP	Lactic acid bacteria
KS-TN8	Stomach	BCP	Lactic acid bacteria
KS-TN9	Stomach	BCP	Lactic acid bacteria
KS-TN10	Stomach	BCP	Lactic acid bacteria
KS-TN11	Stomach	BCP	Lactic acid bacteria
KS-TN12	Stomach	BCP	Lactic acid bacteria
KS-TN13	Stomach	BCP	Lactic acid bacteria
KS-TN14	Stomach	BCP	Lactic acid bacteria
KS-TN15	Stomach	BCP	Lactic acid bacteria
KS-TN16	Stomach	BCP	Lactic acid bacteria
KS-TN17	Stomach	BCP	Lactic acid bacteria
KI-TN18	Intestine	BCP	Lactic acid bacteria
KI-TN19	Intestine	BCP	Lactic acid bacteria
KI-TN20	Intestine	BCP	Lactic acid bacteria
KI-TN21	Intestine	BCP	Lactic acid bacteria
KI-TN22	Intestine	BCP	Lactic acid bacteria
KI-TN23	Intestine	BCP	Lactic acid bacteria
KI-TN24	Intestine	BCP	Lactic acid bacteria
KI-TN25	Intestine	BCP	Lactic acid bacteria
KI-TN26	Intestine	BCP	Lactic acid bacteria
KI-TN27	Intestine	BCP	Lactic acid bacteria
KG-TN28	Gills	BCP	Lactic acid bacteria
KG-TN29	Gills	BCP	Lactic acid bacteria
KG-TN30	Gills	BCP	Lactic acid bacteria
KG-TN31	Gills	BCP	Lactic acid bacteria
KG-TN32	Gills	BCP	Lactic acid bacteria

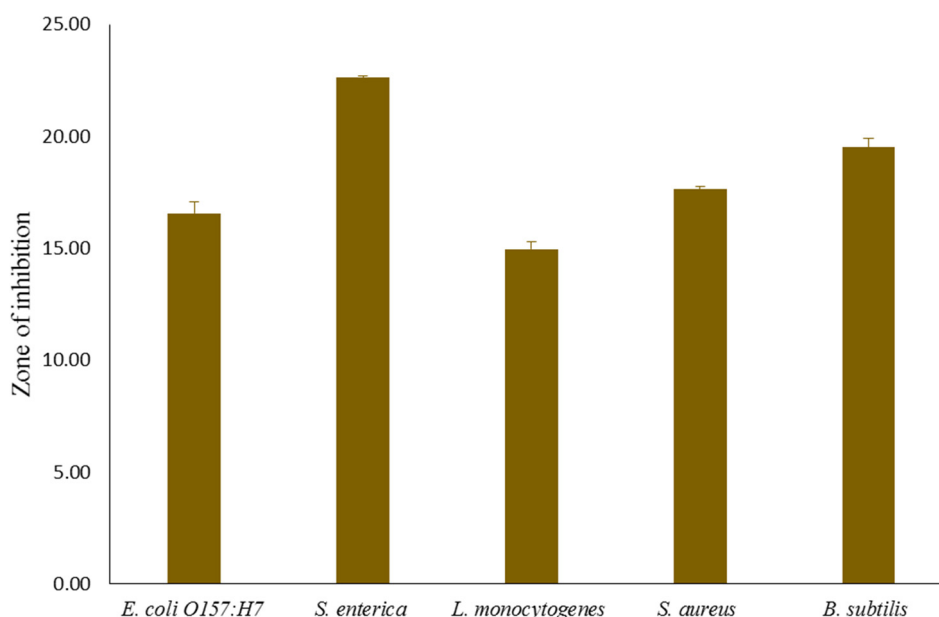


Fig. 1. Antibacterial activity of cell-free-supernatant of *Leuconostoc mesenteroides* KS-TN11 against pathogenic strains.

of colonies by concomitant bacteria and hence recommended for the selective enumeration of the LAB (Bielecka et al., 2000).

In addition, KS-TN11 was further identified by biochemical characterization using API50 kit (Table 2). The strain was identified as a rod-shaped Gram-positive bacterium and showed a close association with *Leuconostoc mesenteroides*. The change in color from violet to yellow in the strip capsule indicated the complete fermentation and KS-TN11 showed utilization of 24 type carbohydrates such as L-arabinose, Ribose-D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, Dulcitol, D-sorbitol, N-acetylglucosamine, Amygdalin, Arbutin, Esculin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, D-raffinose, Gentiobiose, D-turanose, Potassium Gluconate.

Based on the molecular analysis with 16S rRNA gene sequencing, the strain KS-TN11 showed 96% similarity with *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293. The sequence was submitted to GenBank with nucleotide accession number (KY933715). Hence, KS-TN11 was finally confirmed as *Leuconostoc mesenteroides* KS-TN11.

3.4. KS-TN11 inhibits adipocyte differentiation

To evaluate the effect of KS-TN11 on adipogenesis of adipocytes, the 3T3-L1 adipocytes were treated with different concentrations of KS-TN11 (50 mg and 100 mg). Adipogenesis is the diagnostic feature of obese subjects. To this end it was observed that KS-TN11 decreased the lipid content and prevented the accumulation of lipid droplets as depicted by reduced intensity of Oil Red O staining. The effect was not much different at higher and lower dosage of ethanol extract of KS-TN11. However, when compared with con-

trol cells the effect on accumulation of lipid was significantly reduced with KS-TN11 at 100 mg/ml ($P < 0.05$), as shown in Fig. 2.

3.5. α -glucosidase inhibitory activity

The antidiabetic effect of 50 mg and 100 mg of KS-TN11 was evaluated by α -glucosidase assay using p-nitrophenyl- α -D-glucopyranoside (pNPG) as a substrate. The highest inhibition of α -glucosidase was seen with 100 mg/ml sample and minimum inhibition was seen by 50 mg/ml ethanol extract of KS-TN11 as 64% and 41.33%, respectively, as shown in Fig. 3. These results suggest that KS-TN11 as a novel strain with antidiabetic effect.

Aquatic animals have been a great source of lactic acid bacteria (Nair and Surendran, 2005). Interestingly, the majority of the microorganisms isolated from aquatic animals such as fish bear same *Lactobacillus* species as found in animals, including human gastrointestinal tract (Kandler and Weiss, 1986). It has been reported that LAB isolated from fish samples showed potential antibacterial activity against human pathogenic bacteria (Jini et al., 2011). In addition, these fish isolated LAB have gained tremendous attention due to their probiotics functionality. A new probiotic LAB was isolated from *O. niloticus* and the bacterium could be used in aquaculture (Zapata, 2013). There are several other reports suggesting isolation of LAB or probiotic LAB from fish samples; however, the composition changes in each fish samples due to different environmental conditions. In most of the case, the composition remains same that is same LAB were found in a number of different fish samples.

Lactic acid bacteria are known for their beneficial functions including metabolic characteristics and production of biological compounds. A number of LAB isolated from fish showed anti-obesity effects. Bajpai et al. (2016) isolated *Lactobacillus sakei* 111

Table 2
Biochemical characterization of *Leuconostoc mesenteroides* KS-TN11 using API 50 CHL.

Active ingredients	Results	Active ingredients	Results
Glycerol	– ¹	Salicin	–
Erythritol	–	D-Cellobiose	+
D-Arabinose	–	D-Maltose	+
L-Arabinose	+ ²	D-Lactose (bovine origin)	+
D-Ribose	+	D-Melibiose	+
D-Xylose	+	D-Saccharose	+
L-Xylose	–	D-Trehalose	+
D-Adonitol	–	Inulin	–
Methyl- β -D-Xylopyranoside	–	D-Melezitose	–
D-Galactose	+	D-Raffinose	+
D-Glucose	+	Amidon (starch)	–
D-Fructose	+	Glycogen	–
D-Mannose	+	Xylitol	–
L-Sorbose	–	Gentiobiose	+
L-Rhamnose	–	D-Turanose	+
Dulcitol	+	D-Lyxose	–
Inositol	–	D-Tagatose	–
D-Mannitol	–	D-Fucose	–
D-Sorbitol	+	L-Fucose	–
Methyl- α -D-Mannopyranoside	–	D-Arabitol	–
Methyl- α -noside	–	L-Arabitol	–
N-acetylglucosamine	+	Potassium Gluconate	+
Amygdalin	+	Potassium 2-Ketogluconate	–
Arbutin	+	Potassium 5-Ketogluconate	–
Esculin	+		

Note: 24 type carbohydrates: L-arabinose, Ribose-D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, Dulcitol, D-sorbitol, N-acetylglucosamine, Amygdalin, Arbutin, Esculin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, D-raffinose, Gentiobiose, D-turanose, Potassium Gluconate.

¹ The bacterium does not use this carbohydrate.

² The bacterium uses this carbohydrate.

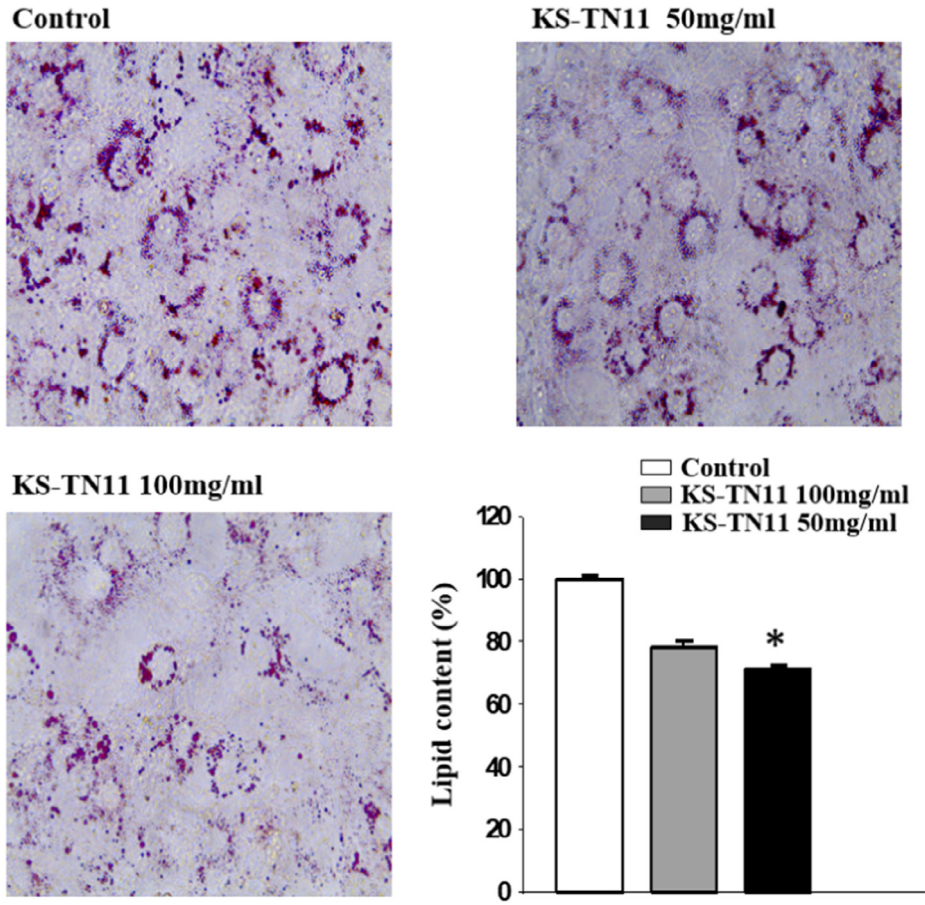


Fig. 2. Effect of *Leuconostoc mesenteroides* KS-TN11 on lipid accumulation in 3T3-L1 adipocytes. * $P < 0.05$ is significant. Analysed by using Student's *t*-test.

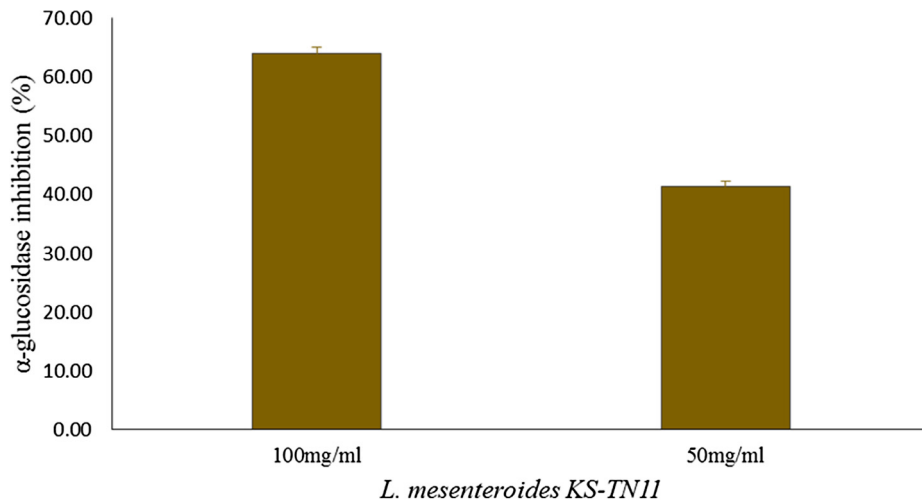


Fig. 3. α -Glucosidase inhibitory activity of *L. mesenteroides* KS-TN11.

from fish showed antidiabetic and antiviral activity. Honda et al. (2012a, 2012b) reported the antidiabetic activity of two lactic acid bacteria strains *Lactobacillus rhamnosus* GG and *Lactobacillus delbrueckii* subsp. *bulgaricus* LB3 in an animal model. However, very less study has been conducted on other LAB species. There are several reports showing the inhibitory effect of α -Glucosidase by dif-

ferent strains of lactic acid bacteria (Ramchandran and Shah, 2008; Michlmayr and Kneifel, 2014; Panwar et al., 2014). Several studies reported the α -glucosidase inhibitory activity of yogurt isolated LAB strains and it was found that strains of *Lactobacillus casei*, *acidophilus*, and *Bifidobacterium* showed approximately 80% of α -glucosidase inhibitory activity (Ramchandran and Shah, 2008).

4. Conclusion

In conclusion our results suggest the antibacterial, and α -glucosidase inhibitory activity of *Leuconostoc mesenteroides* KS-TN11 from Nile Tilapia, *O. niloticus* combined with its possible anti-obesity effect. Thus, KS-TN11's pharmaceutical properties may be explored for therapeutic purposes. However, further studies are required to validate the data and solidify its role.

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Conflict of interest

The authors declare that they do not have any conflict of interest.

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