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SHORT COMMUNICATION

Probiotic sugar confectionery fortified with flax seeds (*Linum usitatissimum* L.)

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Abstract People have affection for confectionary products. Confectionery products are of two types, baker and sugar confectionary. Dark chocolates belong to sugar confectionery class. The present invention was carried out on the preparation of synbiotic dark chocolates. Synbiotics are food products that contain both prebiotics and probiotic microorganisms, wherein prebiotics encourage the growth of probiotics. The synbiotic dark chocolates were amended with flax seeds (*Linum usitatissimum* L.) as a prebiotic for LAB. Flax seed contains fiber and phenolic antioxidants which makes them prebiotic source. The isolated LAB culture, which was identified as *Leuconostoc mesenteroides* based on morphological, biochemical tests and MALDI-TOF, showed the properties of a probiotic culture viz., tolerance to sodium chloride, bile salt, pH, and temperature, sensitivity to antibiotics, nonhemolytic and production of hydrogen peroxide. Cytotoxic activity of the cell free supernatant was assessed against MDA MB 231 and neuroblastoma cell line. Probiotic strain showed 48% and 30% cytotoxicity against MDA MB 231 and neuroblastoma cell line. The synbiotic chocolate was found to have more antioxidant activity, i.e. 90 U/mL by DPPH assay and 200 (µg Trolox/mL) by FRAP assay. The synbiotic chocolate prepared will be beneficial for the gut health of the humans and will also have excellent nutritional value.

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Introduction

The probiotics are live microorganisms which exert a beneficial effect on the health of the host when administered in adequate quantities (FAO/WHO 2002). The development of functional food is done using combination of both prebiotics and probiotics and is called "synbiotic food" (Araujo et al. 2009). LAB are considered as probiotic strains. *Leuconostoc* genus belongs to Firmicutes phylum, are Gram positive, heterofermentative microorganisms, and are present either in coccoid or rod-like shape. *Leuconostoc* sp. has been reported in the fermentation of food like sauerkraut, kimchi and cheese (Jung et al. 2014; Wang et al. 2008). *Leuconostoc* sp. are the bacteria that have significant roles in fermenting foods, such as cheese, kimchi and sauerkraut. *Leuconostoc* has the ability to contribute to other product characteristics, such as flavor, texture and nutritional content (Alegria et al. 2013). The LAB including *Leuconostoc* are recognized as safe (GRAS) hosts for microbial cell factory. Synbiotic affects the host beneficially by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract. Flax seeds contain lignans, α-linolenic acid, and soluble dietary fiber or gum which help in colon cancer prevention and reduce the risk of cardiovascular disease (Hijova et al. 2011). *In-vitro* fermentation of flax seed results in production of high amounts of acetate and propionate (short-chain fatty acids) (Fodje et al. 2009). The combination of fermented flax seed with probiotics helps to enhance the health. Chocolates can be a

suitable vehicle to introduce probiotics and prebiotics in the human diet.

The aim of this work was to formulate a synbiotic chocolate with an added probiotic strain, i.e. *Leuconostoc mesenteroides*.

Materials and methods

Isolation and identification of lactic acid bacteria

Homemade mango pickle was used as a source of LAB. The pickle (1%) was diluted in sterile saline and 100 µL was spread plate on sterile MRS agar medium. The plates were incubated at room temperature for 48 h in the anaerobic jar. The isolates were purified, characterized and identified based on the morphological, biochemical properties and MALDI-TOF (Koubek et al. 2012).

Determination of probiotics potential of isolated *Leuconostoc* sp.

The probiotics potential of isolated *Leuconostoc* sp. was carried by studying the tolerance to NaCl; bile salt; pH; sensitivity to temperature and the antibiotics (Pundir et al. 2013). The potential of isolated *Leuconostoc* sp. to produce lactic acid, hydrogen peroxide and diacetyl was determined as per the protocol of AOAC (1990).

Determination of antioxidant activity of isolated *Leuconostoc* sp.

Ferric reducing ability assay

Antioxidant activity of isolated *Leuconostoc* sp. and cell free supernatant was tested using ferric reducing ability (FRAP) assay (Benzie and Strain 1996). To 0.5 mL of intact cells and cell free supernatant, 2 mL of FRAP reagent [300 mM acetate buffer, pH 3.6] (acetate buffer pH 3.6 composition: 6.4 mL 2 M sodium acetate and 93.6 mL 2 M acetic acid); 10 mM 2, 4, 6-tri (2-pyridyl)-1,3,5-triazine (TPTZ) in 40 mM HCl; and 20 mM ferric chloride were added in the ratio 10:1:1 (v:v:v). The mixture was incubated for 30 min at room temperature in the dark and the absorbance was measured at 593 nm.

DPPH (1, 1-Diphenyl-2-picrylhydrazyl) radical scavenging activity assay

The scavenging effect of the cell free supernatant and isolated *Leuconostoc* sp. was determined using DPPH radical scavenging activity assay. To 0.5 mL of intact cells and cell free supernatant, 2 mL of freshly prepared DPPH solution (0.2 mM) was added. The mixture was vigorously shaken and kept for 30 min in the dark at room temperature. The control sample contained deionized water. The DPPH scavenging activity was monitored by determining the absorbance at 517 nm. The radical scavenging activity was quantified as units/mL (U/mL) by using the following formula (Gazi et al. 2007):

$$\text{DPPH activity (U/mL)} = \frac{(\text{Ab}_c - \text{Ab}_s)}{V} \times 100$$

where Ab_c and Ab_c = absorbance of the control and test samples at 517 nm, respectively, V = volume (mL) of the sample.

Cell surface hydrophobicity

Microbial adherence to hydrocarbons (MATH)

The isolate was grown in MRS broth at 37 °C for 48 h. The broth was centrifuged at 13,000 rpm for 10 min. The pellet was collected; washed twice with phosphate buffered saline of pH 7.4 and resuspended in 3 mL of phosphate buffered saline. The cell suspension (5 mL) was added to 0.6 mL of n-hexadecane and mixed for 2 min. After an incubation period for 1 h at 37 °C, aqueous phase was removed and the absorbance was determined at 560 nm. Percentage of hydrophobicity (H%) was calculated using the equation:

$$\%\text{H} = (\text{Ab0} - \text{Ab1})/\text{Ab0} \times 100$$

The isolates with H% above 50% can be considered as hydrophobic according to Moreira (2005).

Salt aggregation test

The tests was performed as per the protocol of Jonsson and Wadstrom (1984). Bacterial cell suspension was prepared in 0.02 M sodium phosphate buffer (pH 6.8). Bacterial cell suspension (25 µL) was mixed in equal amount with various molar solutions of ammonium sulfate (0.02–4 mol/L). Lowest concentration of ammonium sulphate which showed visible aggregation was scored as the SAT hydrophobicity value.

In-vitro cytotoxicity assay

MTT assay (Sevda et al. 2015).

Leuconostoc mesenteroides were grown in MRS medium for 48 h at 37 °C in anaerobic condition. The cell culture was centrifuged at 5000 rpm for 30 min and supernatant was filtered by cellulose filter paper. The pH was adjusted to 7.0 by NaOH.

Cell line culture

The neuroblastoma and MDA-MB 232 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) F-12, including 10% fetal bovine serum (FBS), 1% (v/v) L-glutamine, 1% (v/v) penicillin–streptomycin solution and 7.5% $NaHCO_3$. The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air and sub cultivated with 70–80% confluence.

Cytotoxicity test

Cell viability were determined by MTT assay. The neuroblastoma and MDA-MB 232 cell lines were incubated in 96 well plate and cultured for 24 h. The cell free supernatant of different concentrations were added into cultured medium. Non-inoculated DMEM medium was used as a negative control. The cell lines were further incubated for 24 h. After incubation, the cell viability was determined by colorimetric MTT assay. MTT solution was added to each well and incubated at 37 °C for 24 h. After incubation, DMSO was added to dissolve the blue colored crystals and absorbance was noted at 570 nm using the microplate reader.

Formulation of synbiotic dark chocolate and its antioxidant properties

The synbiotic chocolate was prepared by incorporating sterile flax seed. The sterilized flax seed (6 g) were inoculated with 10^9 cfu/mL of LAB, incubated for 4 days and added to 125 g of ready to use compound [Morde Food Pvt. Ltd (FCCI), India]. Melted chocolate was molded in the container and kept at 4 °C and 30 °C. The count of *Leuconostoc* sp. in chocolate was determined after every 15 days and bacterial viability was calculated according to the formula:

$$\text{Viability}(\%) = (N/N_0) \times 100\%$$

(Zyzelewicz et al. 2012)

N = log CFU/g after a certain period of storage. N_0 = log CFU/g after product preparation.

Antioxidant activity of flax seeds, dark chocolate and synbiotic chocolate was determined using FRAP and DPPH assay.

Results and discussion

Morphological and biochemical properties of the isolates

Total five isolates were obtained. The isolate No. 3 was selected for further study based on morphological and biochemical characteristics and probiotic potential. The isolate No. 3 was Gram positive rod; catalase negative; oxidase and indole positive. The isolate was found to be nonhemolytic and also ferment glucose and lactose.

MALDI TOF of *Leuconostoc mesenteroides*

The highest intensity peak in *Leu. mesenteroides* appeared at m/z 5123 Da. The species-specific peak masses of *Leuconostoc mesenteroides* are 5123; 6248 and 7116. BioTyper log (score) $\geq$ 2.0 indicates secure genus identification. The MALDI TOF of *Leu. mesenteroides* is shown in Fig. 1.

Probiotics potential of *Leu. mesenteroides*

Leuconostoc mesenteroides showed good tolerance for the NaCl concentration (1–7%); bile salt (0.5–2%), and growth was observed in the MRS broth with p^H 3.0–7.0. The isolate showed good growth in the temperature range 25–40 °C. The LAB was found sensitive to all antibiotics, except Augmentin and Furazolidone. LAB was found to produce 26.75 mg of hydrogen peroxide. The cell free supernatant was found to contain lactic acid. Strains with hydrophobicity values below 40% are hydrophilic (Grajek et al. 2016). In our study, *Leu. mesenteroides* was found to have 7% hydrophobicity to n-hexadecane, which suggests that the strain is hydrophilic. SAT hydrophobicity value of *Leu. mesenteroides* was found to be 1 mol/L.

Antioxidant activity of *Leuconostoc* sp.

Leuconostoc mesenteroides was found to have more antioxidant activity as compared to its cell free supernatant. Antioxidant activity by DPPH method was found to be 60 and 15 U/mL for intact bacteria and its cell free supernatant, whereas antioxidant activity by FRAP assay was 120 and 22 µg trolox/mL for intact bacteria and its cell free supernatant.

Cytotoxicity assay

The MDA-MB-231 cell line is an epithelial, human breast cancer cell line. Neuroblastoma (NB) cell lines are transformed, neural crest derived cells. *In-vitro* cytotoxicity was assessed using MTT reagent which is based on blue formazan product formation due to reduction of MTT by mitochondrial dehydrogenase, which indicates the normal function of mitochondria and cell viability (Lau et al. 2004).

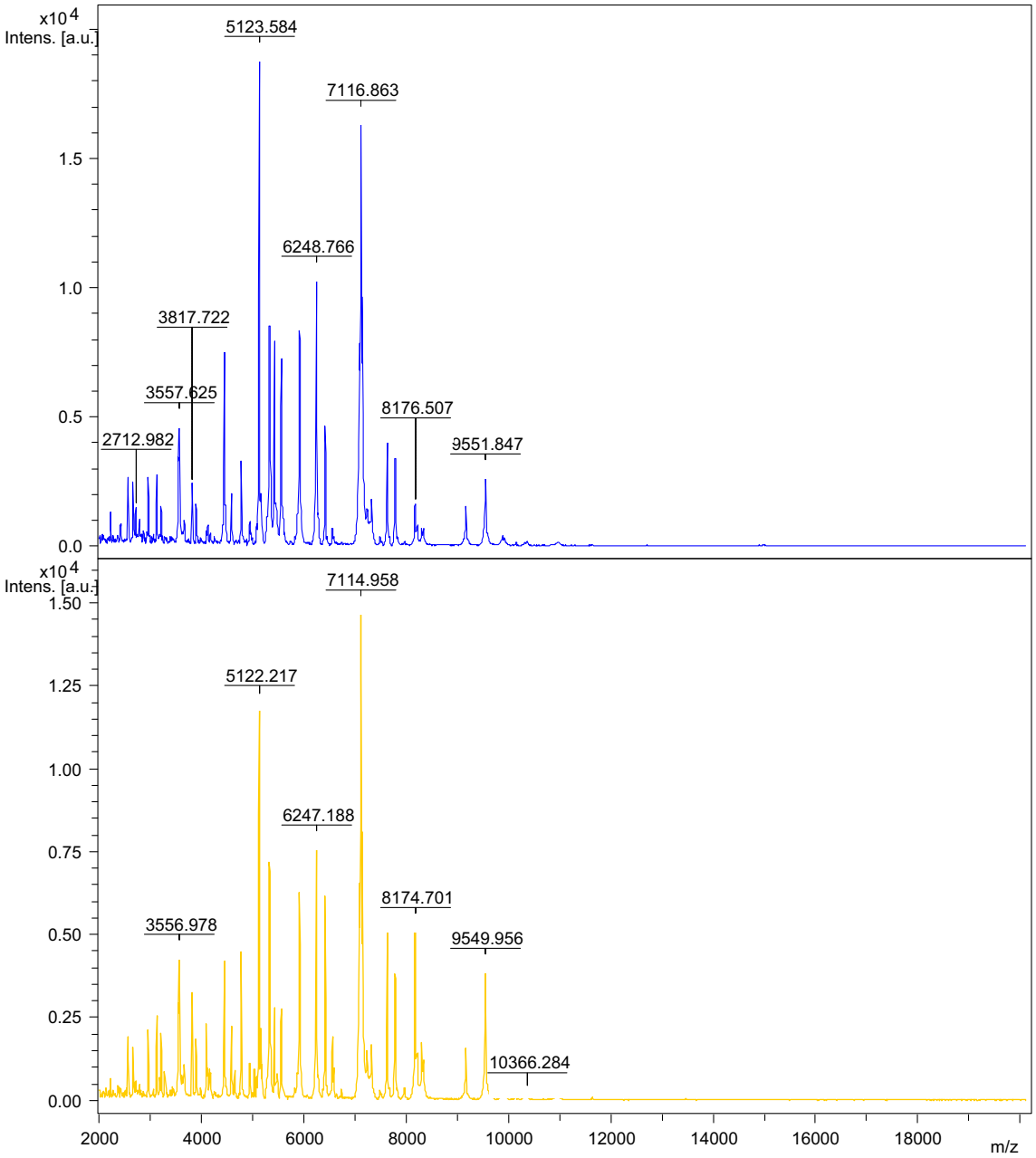

Fig. 1 MALDI-TOF of *Leuconostoc mesenteroides*

MTT assay using MDA-MB 231 cell line

The MTT assay using MDA-MB 231 cell line is shown in Table 1. The strongest effect of cell free supernatant was observed at 50 µL/mL after 24 h of incubation where 48% inhibition was observed (Table 1). IC $_{50}$ is the concentration of the *L. mesenteroides* cell free supernatant to cause 50% reduction in the cell viability. In case of *L. mesenteroides,* the IC_{50} of the cell free supernatant on the MDA-MB 231 cell line was found to be 5%.

MTT assay using neuroblastoma cell line

The MTT assay using Neuroblastoma cell line is shown in Table 1. In these result, 50 µL/mL showed highest inhibition of 30%. In case of cytotoxicity of cell free supernatant of *Leuconostoc* against neuroblastoma cell line, IC_{50} value is more than 5% which showed that the strain will be less effective for antiproliferative activity in neuroblastoma cell line. The cell free supernatant showed more inhibition on MDA-MB cell line at concentration of 50 µL/mL after incubation of 24 h. According to these results, the cell free

Table 1 MTT assay using MDA-MB 231 and Neuroblastoma cell lines

	A_{570} (MDA-MB 231 cell line)	A_{570} (Neuroblastoma Cell line)	Cytotoxicity (%) ± SD (MDA-MB 231 cell line)	Cytotoxicity (%) ± SD (Neuroblastoma cell line)
Control	0.636	0.506	0 ± 0.010	0 ± 0.006
Cell free supernatant (µL/mL)				
0.005	0.555	0.498	12.735 ± 0.007	1.645 ± 0.006
0.05	0.432	0.475	32.023 ± 0.001	6.122 ± 0.003
0.5	0.393	0.421	38.155 ± 0.004	16.721 ± 0.006
5.0	0.374	0.405	41.142 ± 0.01	20.01 ± 0.006
50.0	0.329	0.355	48.270 ± 0.003	29.75 ± 0.006

supernatant can inhibit 48% and 30% of breast cancer and neural diseases respectively.

There is a report on in-vitro cytotoxic activity of probiotic bacterial cell extracts against Caco-2 and HRT-18 colorectal cancer cells (Awaisheh et al. 2016). There is also a report on antioxidant activity of *Lactobacillus pentosus* ITA23 and *Lb. acidipiscis* ITA44 using FRAP method, where the intact cells and cell-free extracts of the two *Lactobacillus* strains showed more than 135 and less than 50 µg trolox/mL of antioxidant activity, respectively (Shokryazdan et al. 2018). Study has been done on *Leuconostoc mesenteroides* NRRL B-1149 as probiotic and its dextran with anticancer properties (Shukla et al. 2014).

Formulation of synbiotic dark chocolate and its antioxidant properties

Leu. mesenteroides ATCC 8293 was investigated for its macromolecular composition and it was found to be 29.7% protein, 7.9% lipid, 24.4% polysaccharide, 2.9% DNA, and 7.4% RNA. The amino acid content in *Leuconostoc mesenteroides* ATCC 8293 has been reported to have large amounts of lysine, glutamic acid, alanine, and leucine (Bang et al. 2017). Soluble dietary fiber (mucilage) of flax seeds has found to increase the growth of lactic acid bacteria in fermented dairy product (HadiNezhad et al. 2013). Flax seed serve as functional ingredient in variety of food products due to its content viz., dietary fiber, lignans, omega-3 and omega-6 essential fatty acids, phytosterols, vitamins and minerals (Teh et al. 2014). *In-vitro* fermentation of flax seed has shown presence of high amounts of acetate and propionate (short-chain fatty acids) (Fodje et al. 2009). The prebiotic content (dietary fiber) of flax seed makes them supporting medium for the growth of *Leuconostoc mesenteroides*. From our results, we conclude the fermented flex seed is suitable as prebiotic. Formulated synbiotic dark chocolates were checked for the viability of probiotic culture. Probiotic culture was found to be viable for six months in synbiotic chocolates when stored at 4 °C

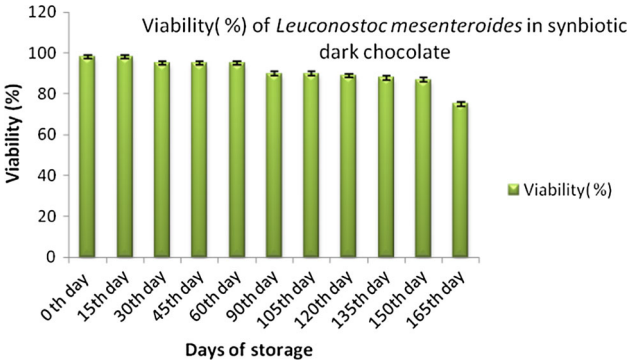

Fig. 2 Viability (%) of *Leuconostoc mesenteroides* in synbiotic dark chocolate stored at 4 °C

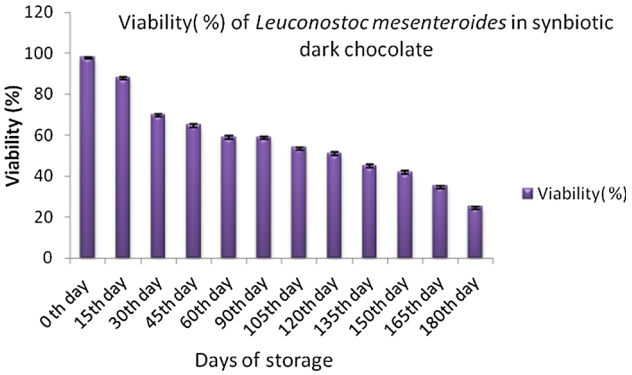

Fig. 3 Viability (%) of *Leuconostoc mesenteroides* in synbiotic dark chocolate stored at 30 °C

(Fig. 2), while viability of the *Leu. mesenteroides* decreased after 15 days in synbiotic dark chocolates when stored at 30 °C (Fig. 3), which is in comparison to the study done by Yao et al. 2008. Therefore, the viability of *L. mesenteroides* was good at 4 °C.

The given growth medium and chocolate components does not inhibit or stop growth of probiotic bacteria. Live form of bacteria can be injected into the intestine of person by using this synbiotic chocolate and main motive of probiotic can be achieved. The DPPH radical scavenging

activity of flax seeds, dark chocolate and synbiotic chocolate was found to be 69, 85 and 90 U/mL, respectively. Antioxidant activity by FRAP assay was found to be 150, 180 and 200 µg trolox/mL. Antioxidant activity of flax seeds is due to phenolic acids, flavonoids, phenylpropanoids and tannins (Kasote 2013). Dark chocolate is enriched with phenolics compounds due to its 67% of cocoa liquor (Da Silva-Madeiros et al. 2015). Synbiotic chocolates was found to have more antioxidant activity due to the combination of natural antioxidant flax seeds, dark chocolate and *Leuconostoc mesenteriodes.*

There is a report on probiotic chocolate prepared using yoghurt powder (Chetana et al. 2013). Also, study is done on preparation of dark chocolate incorporating probiotic *Lb. plantarum* isolated from cocoa beans (Foong et al. 2013). There is a report on sensory quality and volatile profile of dark chocolate enriched with encapsulated probiotic *Lb. plantarum* (Mirkovic et al. 2018).

Conclusion

Chocolate is a preferred choice by people of all ages and it serves as a promising bioactive compound carrier for prebiotic and probiotic.The work presented in this paper, is related with formulation of synbiotic chocolates using fermented flax seed as a prebiotic and probiotic *Leuconostoc mesenteroides.* Nutritional enrichment of food product is the need of hour, which can be accomplished by the consumption of functional foods enriched with dietary fibers. High antioxidant activity of synbiotic chocolates compared to its components, suggests its health beneficiary effects after its consumption. *In-vitro* fermentation of flax seeds has more nutritional values as compared to the original. Further, in vivo studies needs to be carried out to check the effect of these chocolates on the gut microflora. Functional foods will have more market value based on its human benefits. It can be concluded that, the current research will be beneficial for the food industry related with functional food.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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