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Application of Metabolomic Analysis for the Identification of Potential Additional Probiotic Properties of the Bacteriocin Producing *Enterococcus Faecium* ST10Bz Isolated from B*oza*

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Bacteriocin producing strain, *Enterococcus faecium* ST10Bz was isolated from a Bulgarian cereal-based beverage, *boza*. Previous characterization included the characterization of expressed bacteriocin/s, study of potential probiotic properties and safety of the strain. To further characterize the strain, metabolomic analysis of the cell free supernatant obtained from ST10Bz culture was performed. The metabolomic analysis revealed some potentially beneficial properties of the strain, suggesting that this research approach may be applied in screening of probiotic properties and deeper safety characterization of bacterial cultures.
Based on the metabolomic analysis, *E. faecium* ST10Bz was found to produce additional antimicrobials, including lactic acid, β-phenyllactic acid, and phenethylamine, which may play role in the prevention of the colonization in the gut by pathogens, in addition to the previously studied antimicrobial enterocins it could produce. Moreover, increased levels of oleic acid, an anti-inflammatory compound, and γ-hydroxybutyric acid, a precursor of the neurotransmitter GABA, were also recorded. The potential ability of the strain to produce GABA was also supported by the presence of gad gene observed from its bio-molecular analysis of DNA obtained from *E. faecium* ST10Bz. Conversely, xanthine was consumed by *E. faecium* ST10Bz. This purine is considered potentially toxic to humans, thus, the metabolism of it may be considered as beneficial to the host, human and/or other animals. Overall, the *E. faecium* ST10Bz was found out to have additional probiotic properties, particularly expression of metabolites, those that are beneficial to the hosts, as the strain could inhabit the gut. Thus, further studies *in vitro* on the production and quantification of beneficial metabolites must be performed, and *in vivo* animal study as the validation experiment.

1. Introduction

Bacteriocins are antimicrobial proteins produced by different microorganisms, including the lactic acid bacteria (LAB), with inhibitory properties generally against closely related organisms (Chikindas et al., 2018). Production of bacteriocins was considered beneficial property for probiotic organisms, as it may be involved in their competitive ability (Umu et al., 2016) and reducing or complete elimination of pathogens. Apart from bacteriocins, bacteriocinogenic LAB may also produce a variety of metabolites which may be involved in the inhibitory and quorum sensing processes with other microorganisms, as well as interaction with host organisms (Chikindas et al., 2018; Umu et al., 2016).
Enterococcus faecium ST10Bz, a bacteriocin producing strain isolated from boza, a Bulgarian cereal-based beverage, was previously evaluated (Valledor et al., 2020). Previous evaluation included characterization of its bacteriocin production, some additional probiotic properties, and safety of the strain. The aim of present study was to further characterization of the strain, focusing on metabolomic analysis of the cell free supernatant obtained from the culture *of E. faecium* ST10Bz was done with the objective to further evaluate and determine the potential negative and/or additional beneficial properties of studies strain.

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2. Materials and Methods

Previously isolated from boza strain *Enterococcus faecium* ST10Bz and characterized as bacteriocin producers (Valledor et al., 2020) was further investigated in the study.

2.1 Evaluation of bacteriocin production

Cell free supernatant (CFS) was obtained by centrifugation (4,000 × g for 10 min at 4 °C) from 18 h culture of *E. faecium* ST10Bz grown in MRS broth (Difco, Franklin Lakes, NJ, USA) at 37 °C. The resulting CFS was heat-treated for 10 min at 80 °C to deactivate eventually produced proteolytic enzymes and/or H_2O_2 and was filtered using Minisart® 0.22 µm syringe filter (Sartorius AG, Goettingen, Germany). Bacteriocin activity was estimated by serial two-fold dilutions of the treated CFS with 0.1 M potassium phosphate buffer (pH 6.5), evaluated against the test organism *Listeria monocytogenes* ATCC 15313 by spot-in-the-lawn method. The resulting activity was expressed as arbitrary units per milliliter (AU/mL), calculated as shown in eq (1).

$$AU/mL = (D^n)/p \quad (1)$$

where D is the type of dilution, n as the first dilution with no inhibition zone, and p is the volume of the CFS dispensed (Valledor et al., 2020).

2.2 Metabolomic analysis

For the evaluation of the metabolites produced and/or consumed by *E. faecium* ST10Bz, CFS was obtained in the similar manner mentioned above, and the metabolomics analysis was performed according to recommendations of Rhee et al. (2018), as previously applied by Valledor et al. (2022) in evaluation of metabolome analysis of different *Enterococcus* spp. Briefly, 5 mL of *E. faecium* ST1BZ supernatant was homogenized with 5 mL of methanol, supplemented with appropriate internal analytical standard in specific proportions: 10 µL/sample, 1 mg/mL 2-chlorophenylalanine, for an time of 5 min and followed by 5 min sonication at room temperature. The obtained homogenate was centrifuged at 3,000 × g for 5 min and obtained supernatant was filtered via 0.2 µm polytetrafluoroethylene filter. The filtered supernatant (100 µL) was dried on a centrifugal vacuum concentrator (Hanil Scientific Inc., Gimpo, Korea). For GC-TOF-MS (gas chromatography time-of-flight mass spectrometry) analysis, obtained dried sample from previous step was oximated with 40 µL of methoxyamine hydrochloride (20 mg/mL in pyridine) at 30 °C for 120 min, followed by silylation with 40 µL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) at 37 °C for 60 min. The following GC-TOF-MS analysis was conducted on Agilent 7890 gas chromatography system (Agilent Technologies, Palo Alto, CA, USA) linked to an Agilent 7693 auto-sampler (Agilent Technologies) and equipped with a Pegasus BT TOF MS (LECO Corp., St. Joseph, MI, USA) system. A Rtx-5MS column (i.d., 30 m × 0.25 mm, 0.25 µm particle size; Restek Corp., Bellefonte, PA, USA) was used with a constant flow 1.0 mL/min of helium as the carrier gas. Sample (1 µL) was injected into the GC with split in ratio 10:1. The temperature of the oven was maintained at constant 75 °C for 2 min, then incrementally raised by 15 °C/min to 300 °C, and held for 3 min. The temperatures of both, the front inlet and transfer line were 250 °C. The electro-ionization was carried out at -70eV and full scanning over the range of 45 to 600 m/z was used for mass data collection. The obtained GC-TOF-MS raw data was acquired and pre-processed applying the LECO Chroma TOF software (version 5.40, LECO Corp.) and further converted into the NetCDF format (*.cdf) using the LECO Chroma TOF software. After conversion, peak detection, retention time correction, and alignment were processed using the Metalign software package (http://www.metalign.nl). (Valledor et al., 2022).

2.3 D(-) and L(+) lactic acid production

The amount of D(-) and L(+) lactic acid produced by *E. faecium* ST10Bz was determined using D-/L- Lactic Acid (D-/L- Lactate) (Rapid) Assay Kit (Megazyme, Bray, Wicklow, Ireland). This was performed in duplicates using CFS of *E. faecium* ST10Bz obtained as mentioned above.

3. Results and Discussion

Bacteriocin activity against *L. monocytogenes* ATCC15313 was recorded at 19,200 AU/mL, when cultured at 37 °C, an average of several repetitions. Production of bacteriocin with high specific activity against *L. monocytogenes* is considered as positive feature in searching for a suitable antimicrobial for the increasing the food safety and/or alternatives to antibiotics in the treatment of multidrug resistant pathogens (Chikindas et al., 2018; Umu et al., 2017). However, bacteriocins are only one of the several potentially produced antimicrobials

by the LAB and complex approach in the evaluation of the potential antimicrobial properties of probiotic candidates with bacteriocinogenic features needed to cover the deeper metabolic analysis.

Based on the metabolomic analysis, *E. faecium* ST10Bz was found to produce additional antimicrobials, including different organic acids: γ-hydroxybutiric acid (3.09 times increase compared to the control), mevalonic acid (1.62 times increase), oleic acid (3.48 times increase), glyoxylic acid (1.73 times increase), pyruvic acid (5.83 times increase), lactic acid (1.68 times increase), benzoic acid (2.45 times increase), β-phenyllactic acid (12.11 times increase), and phenethylamine (30.26 times increase), which may play role in the prevention of the colonization in the gut by pathogens, in addition to the previously studied antimicrobial enterocins it could produce.

CFS obtained from *E. faecium* ST10BZ, cultured in MRS for 18 h at 37 ºC was analyzed regarding variation in contents of key metabolites, including alcohols, amines, amino acids, carbohydrates, carboxylic acids, fatty acids, lipids, organic acids, phenylpropanoids, purines and pyrimidines (Figure 1). Aim of that part of the research was not only to detect changes and possible production of some antimicrobial metabolites, but to have an overview in a production/consumption of some metabolites with relevance to safety and additional beneficial properties. *E. faecium* ST10BZ showed strain specificity in the production levels of the evaluated metabolites, including increased production, reduced production or consumed as part of anabolic processes. Levels of the tyrosine were reduced by *E. faecium* ST10BZ down to 0.05 times compared to the control, non-fermented MRS. This result can be suggested that correlated to the ability of the strains, as most of *Enterococcus* spp., to produce tyramine, where tyrosine serves as its precursor. However, biogenic amines production was not recorded for *E. faecium* ST10BZ. Moreover, decreased levels of tyrosine in fact means consumption and efficient tyrosine decarboxylase was already associated with treatment of Parkinson disease (Francisco-Donoghue et al., 2014). Moderate reductions of glucose, significant reduction of fructose, stability of lactose and inositos, increase in maltose was recorded in MRS fermented by *E. faecium* ST10BZ (Figure 1), maybe as consequence of preferable catabolite choice by the studied strain.

In general, *E. faecium* ST10BZ can be considered as relatively good producers of different organic and fatty acids (Figure 1). Specific organic acids are regardes as key metabolites from the human and other animals' metabolic pathways, such as glyoxylic, pyruvic, oxalic, malonic, succinic, malic, citric, and glutaric acids, which are intermediates of ATP production (Grkovic et al., 2003) and can be linked to the antimicrobial properties as well (Todorov et al., 2019). Moreover, lactic acid, a typical characteristic metabolite for the LAB was also recorded in increased levels, which could maybe have influence on protein digestion, assimilation of dietary minerals in the gut and antimicrobial properties as well (Rodjan et al., 2017). Increased levels of glyoxylic, pyruvic, malonic acids were recorded compared to the applied control (Figure 1). Additionally, Zhang et al. (2021) suggested and pointed essential role that LAB can metabolize several organic acids, including citrate, lactate and malate, or some amino acids (arginine and glutamine) in order to generate pyruvate and/or ATP, relevant to energetic balance of the cells.

Increased levels of benzoic acid (2.45 times increase) were observed in the CFS from studied strain. Benzoic acid and more specially its salts are used in the food industry as preservative for control of molds, yeasts and some bacteria with influencing anaerobic fermentation of glucose through phosphofructokinase decrease by 95% (Pastrorova et al., 1997). Generally, use for benzoic acid as a preservative in food products is between 0.05–0.1% (Pastrorova et al., 1997). One of the concerns is fact that as most of the chemical preservatives, application of benzoic acid raised some concern related to the observations that benzoic acid, including its salts can be involved in the reactions with ascorbic acid, when applied in some soft drinks and as results may lead to formation of small quantities of carcinogenic products, such as benzene (Yardley-Jones et al., 1991). Benzoic acid is a constituent of pharmaceutical preparations used for the treatment of fungal skin diseases such as tinea, ringworm and athlete's foot. Benzoic acid is a principal component of some topical antiseptics and inhalant decongestants with a long history of use. In the early 20th century benzoic acid was used as an expectorant, analgetic and antipyretic (Caroline et al., 2019).

Levels of xanthine were reduced by the studied *E. faecium* ST10Bz. Xanthine is belonged to the group of purines, and in case of increased levels, may have toxic effect and can cause xanthine stones on patients with severe hyperuricemia (Eldho et al., 2017). Can be speculated that studied strain can be applied as beneficial strains for individuals exposed to xanthine, to eliminate the toxicity it imposes.

	C	ST10Bz	Tentative metabolites
Alcohols	1	0.93	Ethylene glycol
	1	1.24	Propylene glycol
	1	1.37	2,3-Butanediol
	1	0.91	1,3-Propanediol
	1	1.25	1,4-Butanediol
	1	1.13	Erythritol
	1	1.07	Pinitol
Amines	1	1.01	Methylamine
	1	1.18	Ethanolamine
Amino Acids	1	1.05	Alanine
	1	0.96	Glycine
	1	0.98	Leucine
	1	1.18	Valine
	1	1.16	2-Methylserine
	1	1.09	Isoleucine
	1	1.13	Proline
	1	0.98	Glycine
	1	0.99	Serine
	1	1.01	Threonine
	1	0.81	Aspartic acid
	1	0.93	β-Alanine
	1	1.08	Homoserine
	1	1.34	Methionine
	1	1.1	Pyroglutamic acid
	1	0.94	γ-Aminobutyric acid
	1	0.82	Phenylalanine
	1	0.98	Glutamic acid
	1	0.66	Homocysteine
	1	0.97	Asparagine
	1	2.97	Ornithine
	1	1.77	Histidine
	1	0.05	Tyrosine
	1	1.03	Tryptophan
Carbohydrates	1	1.05	Glycerol
	1	0.45	Glyceric acid
	1	0.42	Threonic acid
	1	0.79	Xylitol
	1	0.17	Fructose
	1	0.91	Glucose
	1	0.92	Galactaric acid
	1	1	Myo-Inositol
	1	1.08	Lactose
	1	8.05	Maltose
	1	1.2	2-Furoic acid
	1	0.97	Phosphoric acid

	C	ST10Bz	Tentative metabolites
Fatty Acids	1	0.64	2-Methylbutyric acid
	1	3.09	γ-Hydroxybutyric acid
	1	0.45	Pelargonic acid
	1	0.74	2,4-Dihydroxybutanoic acid
	1	1.62	Mevalonic acid
	1	1.07	Palmitic acid
	1	3.48	Oleic acid
	1	1.31	Arachidic acid
Lipids	1	1.22	α-Glycerophosphoric acid
	1	1.24	1-Monomyristin
	1	1.55	Oleamide
	1	0.97	1-Monopalmitin
Organic Acids	1	1.73	Glyoxylic acid
	1	5.83	Pyruvic acid
	1	1.68	Lactic acid
	1	0.72	Glycolic acid
	1	0.56	Oxalic acid
	1	0.76	Hydracrylic acid
	1	1.47	Malonic acid
	1	0.99	Urea
	1	1.01	Succinic acid
	1	0.79	3,4-Dihydroxybutyric acid
	1	0.78	Aminomalonic acid
	1	0.91	Malic acid
	1	0.93	Pipecolic acid
	1	0.99	Citric acid
	1	0.97	Glutaric acid
Phenylpropanoids	1	2.45	Benzoic acid
	1	0.61	Phenylacetic acid
	1	30.26	Phenethylamine
	1	12.11	β-Phenyllactic acid
	1	0.73	4-Hydroxyphenylacetic acid
Purines	1	0.76	Hypoxanthine
	1	2.3	Adenine
	1	0.03	Xanthine
	1	1.04	Guanine
	1	1.1	Inosine
	1	1.38	Guanosine
	1	2.18	Adenosine
Pyrimidines	1	0.35	Uracil
	1	2.79	Thymine
	1	0.79	Orotic Acid
	1	0.49	Uridine

Figure 1: Heat map analysis of the tentative metabolites produced by *E. faecium* ST10Bz (ST10Bz), normalized by the control (C; MRS broth).

Production of β-phenyllactic acid was clearly recorded for *E. faecium* ST10Bz with increase of 12.11 times compared to the control. This compound is considered as potent antimicrobial with activity against some molds (Ning et al., 2017). Application of β-phenyllactic produced by *Lactobacillus plantarum* against different representatives of *Aspergillus*, *Penicillium*, and *Fusarium* was previously investigated by Lavermicocca et al. (2003).

Increased levels of α-hydroxybutyric acid (α-hydroxybutanoic acid), 3.09 times compared to the control were recorded (Figure 1). Moreover, 2,4-dihydroxybutanoic acid was reduced (0.74 times decrease), but phenethylamine was increased (30.26 times increase) (Figure 1). Mentioned metabolites are mainly involved in energy metabolism, when bacterial cultures are exposed to oxidative stress and may have role in the active defense response of the bacteria (Zhang et al., 2021). Moreover, increased levels of oleic acid, an anti-inflammatory compound, and *E. faecium* ST10Bz**.** GABA is a bioactive amine, non-protein amino acid, synthesized by plants, animals and microorganisms from the decarboxylation of L-glutamate and its derivatives mainly, with alternative routes that vary according to the need and organism. The deficiency of GABA can have role in neurological diseases, including etiology of schizophrenia (Wu and Sun, 2015). GABA was reported as anti-hypertensive and antidepressant activities bioactive molecule (Cui et al., 2020). As a supplement it can play an modulating role in behaviour, cognition, and the response of the body to stress factors. GABA may improve sleeplessness and depression (Okada et al., 2000; Abdou et al., 2006), enhancing immunity (Abdou et al., 2006), relieving anxiety and menopausal syndrome (Wong et al., 2003), regulating blood pressure (Hayakawa et al., 2004), fighting obesity (Oh et al., 2005), and improving visual cortical function (Leventhal et al., 2003).

Lactic acid bacteria, including *Enterococcus* spp. are known to produce lactic acid as final metabolite of the carbohydrate digestion. *Enterococcus* spp. are known to produce predominant L(+)-lactic acid, with some traces of D(-)-lactic acid. However, some species as *Lactobacillus delbrueckii* subsp. *bulgaricus* and all *Leuconostoc* species, are known as D(-)-lactic acid producers. High intake of D(-)-lactic acid can be associated with acidosis, especially in infants and may be related with some chronic diseases (Vitetta et al., 2017). For *E. faecium* ST10Bz the amounts D(-)- and L(+)-lactic acids produced were: 0.581 g/L and 15.302 g/L respectively. Similar ratios between both isomers were as well reported by Bhagwat and Annapure (2019), stated values between 5-12 g/L for total lactic acid produced by different *Enterococcus* spp. strains.

4. Conclusion

Overall, the ***E. faecium* ST10Bz** was found out to have additional probiotic properties, particularly expression of several metabolites that may be beneficial to the hosts, as the strain could inhabit the gut. In addition to bacteriocin/s produced by *E. faecium* ST10Bz additional antimicrobial metabolites were also recorded, with some previously reported as antifungal. Thus, further studies *in vitro* on the production and quantification of beneficial metabolites must be performed, as well as validation through *in vivo* animal study.

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