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Research article

ISOLATION, IDENTIFICATION AND CHARACTERISTICS OF *HEYNDRICKXIA COAGULANS* STRAINS FOR INCLUSION IN PROBIOTIC PREPARATIONS

Yordanka Gaytanska[1], Bogdan Goranov[1], Rositsa Denkova-Kostova[2], Denica Blazheva[1]✉, Petya Ivanova[2]

[1] Department of Microbiology and Biotechnology, Technological Faculty, University of Food Technologies, Plovdiv, Bulgaria

[2]Department of Biochemistry and Nutrition, Technological Faculty, University of Food Technologies, Plovdiv, Bulgaria

✉ Corresponding author: d_blazheva@uft-plovdiv.bg

ORCID Number: 0000-0002-4939-881X,

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Abstract

The inclusion of spore-forming bacteria in probiotic preparations for human and veterinary applications is a new trend in food supplements. The objective of this study was to isolate, identify and characterize the biological activities of two novel strains for the purpose of inclusion in probiotic supplements for animal feed, for food supplements for humans, as well as in the composition of plant protection preparations. The two strains were identified as *Heyndrickxia coagulans* through phenotypic and molecular genetic methods. Both strains were investigated for their total phenolics contents and antioxidant activity (DPPH and FRAP methods). In a series of experiments, the antibacterial and antifungal activity of the biomass and the cell-free supernatant of the cultural medium of *Heyndrickxia coagulans* after the cultivation of the strains in three different cultural media were determined. They showed significant difference in their antioxidant activity ($p>0.05$) and their antimicrobial activity against *Escherichia coli, Salmonella enterica* subsp. *enterica* serovar Enteritidis, *Salmonella enterica* subsp. *enterica* serotype Abony, *Staphylococcus aureus, Listeria monocytogenes, Pseudomonas aeruginosa, Bacillus cereus, Candida utilis, Saccharomyces cerevisiae, Aspergillus niger, Penicillium chrysogenum, Aspergillus flavus, Fusarium moniliforme*. It was established that one of the strains exhibited clear potential for inclusion in probiotic and plant protection preparations.

1. Introduction

Spore-forming bacteria are used as probiotic additives in animal feed, in human food supplements, and in registered drugs (Casula *et al.*, 2002; Bomko *et al.*, 2016; Cao *et al.*, 2020). Their high heat resistance and ability

to survive as spores in the adverse conditions of the gastrointestinal tract make them suitable as food supplements. This is a new direction in the science of probiotics for human and veterinary purposes (Hong *et al.*, 2005; Cao *et al.*, 2020). Bacterial spores are formed in nature as a means of survival under extreme environmental conditions that could destroy the vegetative bacterial cells (Nicholson *et al.*, 2000). Under harsh conditions, most often depletion of nutrients in the immediate vicinity of the living cell, the bacteria stop their growth and undergo a process of sporulation (Errington, 2003).

One of the most promising spore-forming bacteria with probiotic potential are *Heyndrickxia coagulans* (formerly *Bacillus coagulans*). Their spores tolerate technological processes well, and when they enter the duodenum, they germinate and the resulting vegetative cells populate the intestinal tract of humans and animals. Due to their high biological activity, the spores of this species are included in the composition of pharmaceutical preparations (Hoa *et al.*, 2000; Bomko *et al.*, 2017; Cao *et al.,* 2020).

Extracts derived from bacilli have been shown to have antimicrobial, antifungal, antioxidant, and anticancer activities (Blunt *et al.*, 2018; León *et al.*, 2010; Prazdnova *et al.*, 2015; Velasquez Cardona *et al.*, 2018). Since oxidative stress is directly or indirectly involved in various pathological conditions in humans (Forman and Zhang, 2021), bioproducts derived from *Bacillus* sp. may hold promise as effective agents for the prevention and treatment of chronic diseases by reducing oxidative stress, including cancer (Céspedes *et al.,* 2023). Synthetic antioxidants, which are widely used in industrial applications, are being investigated for their toxicity and carcinogenic effects (Moktan *et al.*, 2008; Thitilertdecha *et al*., 2008). Interest in finding natural antioxidant agents with low cytotoxicity has increased significantly (Thitilertdecha et al., 2008). Plants are mainly used for their production (Teow *et al*., 2007; Erkan *et al*., 2008), but microbial sources have shown potential for the production of natural antioxidants in various fermented products (Sheih *et al*., 2000; Wang *et al*., 2007; Esaki *et al*., 1997; Hirota *et al*., 2000; Ren *et al*., 2006; Yen *et al*., 2003). Kumari *et al*. (2012) also demonstrated that microorganisms can produce antioxidants and these antioxidants act as preservatives in food products.

Bacteria of the former genus *Bacillus* (including *Heyndrickxia coagulans)* exhibit different antimicrobial activities, associated with the formation of more than 200 antibiotic substances (Khochamit *et al*.,2015; Tenea *et al*., 2022), which is why individual strains differ in their antagonistic spectrum (Hoa *et al*., 2000; Tenea *et al*., 2022). This requires the selection of strains with pronounced antimicrobial activity against pathogenic and saprophytic microorganisms.

Bacilli with proven antifungal and antibacterial properties, are a part of biological preparations both for agriculture (as plant protection products, for example, phytosporin and probiotics for animal husbandry - biosporin) and for human purposes (Tenea *et al*., 2022). The activity of these forms depends on the concentration of substances with antimicrobial activity produced by the selected strains during cultivation, as well as on the amount of spores obtained. The type and concentration of substances with antimicrobial activity and the amount of spores depend on the composition of the fermentation medium and the cultivation conditions. In order for a preparation to be used in practice, it is necessary to contain a high concentration of spores of the respective strain (not less than 10^9 cfu/g).

The aim of the present study was to isolate, identify and characterize the biological activities of two *Heyndrickxia coagulans* strains, which are requirements for probiotic bacteria.

2. Materials and methods

2.1. Microorganisms

The subjects of the study are two unidentified bacterial strains, isolated from spontaneously coagulated pasteurized milk. The strains were maintained on nutrient medium with the following composition

(g/dm3): peptone from meat – 10; NaCl – 5; meat extract – 3; glucose – 10; agar-agar – 15, pH 7.5, and subcultured every 60 days.

2.2. Methods

2.2.1 Phenotypic identification

The phenotypic identification was done using API 50 CH and API 50 CHB/E medium (BioMérieux® SA, Marcy-l'Etoile, France) following the manufacturer's manual.

2.2.2 Molecular identification

The molecular identification of the new strains was performed according to the method of Urshev *et al.* (2024).

2.2.3 Cultivation of Heyndrickxia coagulans

The isolates were cultivated in the following nutrient media:

Medium A (g/dm^3): molasses – 20; peptone – 10; corn extract – 3; $CaCl_2$ – 0.22; $MgSO_4$ – 0.11; K_2HPO_4 – 0.24; pH 7.5

Medium B (g/dm^3): malt – 20; corn extract – 3; molasses – 20; CaCl2 – 0.22; $MgSO_4$ – 0.11; K_2HPO_4 – 0.24; pH 7.5

Medium C (g/dm^3): peptone – 10; NaCl – 10; meat extract – 5; $CuCl_2$ – 0.001; $MgSO_4$ – 0.5; pH 7.5

The cultivation of the strains was carried out in 500 cm^3 Erlenmeyer flasks with 100 cm^3 nutrient medium at a temperature of 37°C for 48 hours on a rotary shaker (220 min^{-1}). The nutrient medium was inoculated with 1% (v/v) of 18 h vegetative cell suspension.

2.2.4 Total polyphenolics assay

The content of total polyphenolics in the samples was determined according to the method of Ainsworth and Gillespie (2007) with Folin-Ciocalteu (FC) reagent, briefly - 0.2 mL of the sample was mixed with 1.0 mL of FC reagent and after 30 sec, 0.8 mL of 7.5% Na_2CO_3 was added. After 30 min in the dark, the absorbance was measured at 765 nm. The results are presented as mg gallic acid equivalents (GAE) in 1 mL (mg GAE/mL).

2.2.5 DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

The radical scavenging activity of the samples was determined according to a modified method of Dimov *et al.* (2018) as follows: 0.15 mL of the sample was mixed with 2.85 mL of freshly prepared DPPH solution (0.06 mM in 96% ethanol). After 30 minutes in the dark at room temperature (23-25°C) the absorbance was measured at 517 nm. The results are presented as mM Trolox equivalents (TE) in 1 mL (mM TE/mL). For comparison, the synthetic antioxidant butylhydroxytoluene (BHT) was used in concentrations of 0.01-0.1 % (positive control).

2.2.6 FRAP (ferric reducing antioxidant power) assay

The metal reducing activity of the samples was determined according to the following modification of the method of Dimov *et al.* (2018): 0.1 of sample was added to 3 mL of freshly prepared FRAP reagent [0.3 M acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in a ratio of 10:1:1]. The reaction mixture was incubated for 10 minutes at 37°C in the dark and the absorbance was measured at 593 nm. $FeSO_4$.$7H_2O$ was used to construct a standard line. The results are presented as µmol Fe^{2+} equivalents in 1 mL (µmol Fe^{2+}/mL). For comparison, the synthetic antioxidant BHT was used in concentrations of 0.01-0.1% (positive control).

2.2.7 Determination of antimicrobial activity

To determine the antimicrobial activity of the tested strains against pathogenic and saprophytic microorganisms, biomass in saline (BM) and cell-free supernatant (CFS) were obtained from a 24-hour culture of the strains. The culture medium was subjected to centrifugation at 3500 min^{-1} for 15 min to separate the biomass, after which the cell-free supernatant was separated and filtered through a membrane filter (0.45 µm). The biomass was washed twice with saline and brought to the initial volume with saline.

The antimicrobial activity was tested against *Escherichia coli* ATCC 25922, *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 25928, *Salmonella enterica* subsp. *enterica* serotype Abony NTCC 6017, *Staphylococcus aureus* ATCC 6538P, *Listeria monocytogenes* ATCC 19111, *Pseudomonas aeruginosa* ATCC 9027, *Bacillus cereus* ATCC 14579, *Candida utilis* ATCC 42402,

Saccharomyces cerevisiae ATCC 9763, *Aspergillus niger* ATCC 1015, *Penicillium chrysogenum* ATCC 28089, *Aspergillus flavus* ATCC 9643, *Fusarium moniliforme* ATCC 38932. Suspensions of each of the test-microorganisms, or their spores for the molds, (10^6-10^7 cfu/cm^3), were prepared and used to inoculate Petri dishes with LBG-agar medium (Composition (g/dm^3): tryptone - 10; yeast extract - 5; NaCl - 10; glucose – 10; agar - 15, pH 7.5). After solidification of the agar, wells (6 mm in diameter) were prepared and 0.06 cm^3 of BM or CFS were pipetted into the wells and the Petri dishes were incubated at 30°C for saprophytic microorganisms or 37°C for pathogenic microorganisms for 24 to 48 h. The antimicrobial activity was determined by measuring the zones of inhibition in mm.

2.3. Data analysis

All experiments were performed with three replications (n=3) and the results were presented as mean values. The obtained results were analysed using Statgraphics Centurion 18 (Statgraphics Technologies, Inc.) by the method of one-way analysis of variance (ANOVA) according to Duncan's test with the probability of accepting the null hypothesis ($p < 0.05$).

3. Results and discussions

Two novel strains were isolated form spontaneously fermented pasteurized milk. Their characterization began with an assessment of the purity of the culture, macroscopic and microscopic morphological control. The colony and cell morphology of the isolates are presented in Tables 1 and 2.

When cultivated on meat extract agar, the isolated strains grew in the form of round to elliptical whitish colonies with lobate edges and a dry texture, which were easily separated from the medium surface. Further microscopic investigations showed that both strains were Gram-positive (Gr (+)), spore-forming rods.

Table 1. Colony morphology of the novel strains

Strain	Shape	Edges	Surface	Elevation	Texture	Colour	Size
M	circular to elliptical	lobate	smooth	raised	dry	whitish	3 – 4 mm medium
BJ	circular to elliptical	lobate	smooth	raised	dry	whitish	3 – 4 mm medium

Table 2. Cell morphology of the novel strains

Strain	Shape	Edges	Spatial arrangement	Motility	Spore formation	Gram staining
M	short fine rods	round-ended	solitary, side-by-side or short chains	yes	yes	Gr (+)
BJ	short fine rods	round-ended	solitary, side-by-side or short chains	yes	yes	Gr (+)

These characteristics necessitated the use of the API 50 CHB/E rapid identification system for representatives of *Bacillaceae.* The ability of the isolated strains to utilize 49 carbon sources included in the system was investigated (Table 3). After processing of the test results with apiweb®, the strains were identified with the corresponding percentage of reliability.

Table 3. Carbohydrate utilization patterns of the tested isolates

Carbohydrate	Strain	
	M	**BJ**
Control	-	-
Glycerol	+	+
Erythriol	-	-
D-arabinose	-	-
L-arabinose	+	+
Ribose	+	+
D-xylose	+	+
L-xylose	-	-
Adonitol	-	-
β-metyl-D-xyloside	-	-
Galactose	+	+
D-glucose	+	+
D-fructose	+	+
D-mannose	+	+
L-sorbose	-	-
Rhamnose	-	+
Dulcitol	-	-
Inositol	-	-
Manitol	+	+
Sorbitol	+	+
α-methyl-D-mannoside	+	-
α-methyl-D-glucoside	+	+
N-acetyl-glucosamine	+	-
Amigdalin	+	+
Arbutin	+	+
Esculin	+	+
Salicin	+	+
Cellobiose	+	+
Maltose	+	+
Lactose	+	+
Melibiose	+	+
Saccharose	+	+
Trehalose	+	+
Inulin	+	+
Melezitose	+	+
D-raffinose	+	+
Amidon	+	-
Glycogen	+	+
Xylitol	-	-
β-gentiobiose	+	+
D-turanose	+	-
D-lyxose	-	-
D-tagarose	-	-

D-fuccose	-	-
L-fuccose	-	-
D-arabitol	-	-
L-arabitol	-	-
Gluconate	+	-
2-keto-gluconate	-	-
5-keto-gluconate	-	-
Identification	*Heyndrickxia coagulans*	*Heyndrickxia coagulans*
Reliability, %	93,9	91,8

After the data from Table 3 was processed with the apiweb® software both strains M and BJ were determined as *Heyndrickxia coagulans* with a reliability of 93.9% and 91.8%, respectively. For more accurate species determination, molecular genetic methods were used. The comparative analysis of the 16S rDNA gene sequence confirmed the identification of the strains – strain M was assigned to *Heyndrickxia coagulans* with a percentage of confidence between its nucleotide sequence of the 16S rDNA and the partial sequence of the 16S rDNA of *Heyndrickxia coagulans* DSM 1 = ATCC 7050 of 99%, strain BJ was assigned to *Heyndrickxia coagulans* with a percentage of confidence between its nucleotide sequence and the partial sequence of the 16S rDNA of *Heyndrickxia coagulans* NBRC 12583 of 99 %.

The former genus *Bacillus* is well known for its production of metabolites with antioxidant activity, such as phenolic acids (Safronova *et al*., 2021). Polyphenols exhibit strong antioxidant activity, which is closely related to their high reactivity towards reactive oxygen species. The isolates identified as *H. coagulans* M and *H. coagulans* BJ showed similar phenolic content (Table 4).

Table 4. Antioxidant activity of the *Heyndrickxia coagulans* strains

DPPH, mM TE/cm^3		FRAP, µmol Fe^{2+}/cm^3		Total polyphenols, mg GAE/mL	
H. coagulans M	*H. coagulans* BJ	*H. coagulans* M	*H. coagulans* BJ	*H. coagulans* M	*H. coagulans* BJ
0.38±0.01^a	0.56±0.01^b	0.54±0.02^a	0.80±0.02^b	0.21±0.01^a	0.23±0.02^a

$^{a, b}$ - indices showing significant differences ($p < 0.05$) between the mean values in the rows for each method

Most antioxidant compounds were polyphenols, acting as reducing agents (free radical scavengers), metal chelators and singlet oxygen scavengers (Mathew and Abraham, 2006). This was also evidenced by the results form determining the antioxidant activity in the present study. *H. coagulans* BJ showed higher antioxidant activity than *H. coagulans* M, both in the DPPH and FRAP assays (Table 4). Such positive correlations between antioxidant activity and total phenolic content have been demonstrated for a number of foods and beverages - red wines (Vinson and Huntz, 1995), vegetables (Kaur and Kapoor, 2002; Ordoñez *et al*., 2006), grapes, marc, must, wine and juice (Yildirim *et al*., 2005), in some medicinal and aromatic plants (Miliauskas *et al*., 2004). This indicates that polyphenols in our samples can play the role of electron and hydrogen donors.

In order to compare the antioxidant activity the *H. coagulans* strains with the activity of a synthetic antioxidant, the activity of BHT in concentrations of 0.005-0.1 % according to the DPPH and FRAP methods was determined. The results shown in Table 5 indicate that with

increasing the BHT concentration, the antioxidant activity increased proportionally. In the DPPH method, the antioxidant activity values for the two strains ranged within 0.38-0.56 mM TE/cm^3, and at the studied BHT concentrations – within 0.15-0.68mM TE/cm^3, i.e. in this method the results obtained in the present research were comparable to those obtained with the synthetic antioxidant up to a concentration of 0.03%.

Table 5. Antioxidant activity of BHT

Concentration of BHT, %	DPPH, mM TE/mL	FRAP, µmol Fe^{2+}/mL
0.005	0.15±0.00	0.22±0.02
0.01	0.30±0.01	0.48±0.02
0.02	0.46±0.03	0.86±0.03
0.03	0.55±0.03	1.05±0.01
0.04	0.63±0.01	1.35±0.01
0.05	0.67±0.01	1.65±0.02
0.10	0.68±0.02	2.17±0.02

In the FRAP method, BHT showed values ranging between 0.22 µM Fe^{2+}/cm^3 and 2.17 µM Fe^{2+}/cm^3 at the tested concentrations, while our samples had values of 0.54-0.80 µM Fe^{2+}/cm^3, i.e. the extracts showed the ability to donate hydrogen atoms and therefore can serve as free radical "scavengers", acting as primary antioxidants (Chung *et al*., 2006).

In a series of experiments, the antimicrobial activity of the *Heyndrickxia coagulans* strains was studied after their cultivation in three different fermentation media - medium A, medium B and medium C. The influence of the composition of the nutrient medium on the antibacterial and antifungal activity of the biomass and cell-free supernatant was established (Table 6). *H. coagulans* M cultivated on medium A did not exhibit antifungal activity against *Aspergillus flavus* and displayed a less pronounced one against *Penicillium chrysogenum* (Table 6). For the remaining yeasts and molds, a strongly pronounced antifungal activity was observed, which was evidenced by the large diameter of the inhibition zones, ranging from 14 mm to 32 mm. The cell-free supernatant of the strain cultivated in medium A showed antifungal activity against all tested molds, with the diameter of the inhibition zones varying from 10 mm to 32 mm. The biomass of the strain cultivated in medium B also showed lower activity against *Aspergillus flavus*, as well as a less pronounced one against *Penicillium chrysogenum*, where the smallest inhibition zones were established - 9 mm. For all other tested molds and yeasts, a highly pronounced antifungal activity was observed, characterized by inhibition zones ranging from 13 mm to 28 mm. The supernatant obtained from the cultivation of the studied strain in medium B also exhibited high activity against the tested microorganisms, with inhibition zones ranging from 10 mm to 34 mm. Unlike the previous two media, in medium C, a highly pronounced antifungal activity was observed, both in the biomass and in the cell-free supernatant against all tested representatives of molds and yeasts, with the inhibition zones ranging from 12 mm to 37 mm for the different test-microorganisms.

From the data presented in Table 6, it is clear that the biomass of *H. coagulans* M, cultivated in all media exhibited weak antibacterial effect on the growth of *Salmonella enterica* subsp. *enterica* serovar Enteritidis, which was confirmed by the small diameter of the inhibition zones (8 mm). The same result was observed for the activity of the cell-free supernatant against this test-microorganism.

Table 6. Antimicrobial activity of the *Heyndrickxia coagulans* strains

Test-microorganism	*Heyndrickxia coagulans* M						*Heyndrickxia coagulans* BJ					
	Medium A		**Medium B**		**Medium C**		**Medium A**		**Medium B**		**Medium C**	
	BM	CFS	BM	CFS	BM	CFS	BM	CFS	BM	CFS	BM	CFS
***C. utilis* ATCC 42402**	20.33±0.47	20.17±0.24	25.17±0.24	25.33±0.47	37.50±0.41	32.33±0.47	-	-	-	-	-	-
***S. cerevisiae* ATCC 9763**	32.67±0.47	30.17±0.24	28.33±0.47	34.67±0.47	30.67±0.47	24.17±0.24	-	-	-	-	-	-
***A. niger* ATCC 1015**	17.50±0.41	15.17±0.24	13.17±0.24	12.33±0.47	22.17±0.24	22.33±0.47	10.17±0.11	9.17±0.12	9.17±0.45	9,17±0.16	11.17±0.34	9.17±0.29
***A. flavus* ATCC 9643**	-	10.17±0.24	9.17±0.24	10.50±0.41	37.67±0.47	25.67±0.47	9.23±0.22	-	15.24±0.27	10.17±0.33	15.20±0.12	14.11±0.37
***F. moniliforme* ATCC 38932**	28.50±0.41	32.33±0.47	20.33±0.47	33.67±0.47	20.17±0.24	12.17±0.24	9.12±0.34	10.15±0.17	10.14±0.33	9.13±0.42	12.12±0.44	10.17±0.14
***P. chrysogenum* ATCC 28089**	9.17±0.24	10.33±0.47	9.33±0.47	10.17±0.24	15.33±0.47	12.33±0.47	-	-	12.17±0.12	10.15±0.00	10.14±0.22	9.2±0.26
***E. coli* ATCC 25922**	15,33±0,47	17,33±0,47	13,33±0,47	18,50±0,41	13,33±0,47	9,17±0,24	-	-	-	-	-	-
***S. enterica* subsp. *enterica* serovar. Enteritidis ATCC 25928**	8,17±0,24	8,33±0,47	8,17±0,24	8,17±0,24	8,17±0,24	-	-	-	-	-	-	-
***S. enetrica* subsp. *enterica* serotype Abony NTCC 6017**	15,50±0,41	10,33±0,47	9,33±0,47	9,33±0,47	8,33±0,47	-	-	-	-	-	-	-
***S. aureus* ATCC 6538P**	24,67±0,47	24,67±0,47	17,50±0,41	33,67±0,47	32,67±0,47	15,50±0,41	-	-	-	-	-	-
***P. aeruginosa* ATCC 9027**	10,17±0,24	14,67±0,47	13,17±0,24	16,33±0,47	17,50±0,41	9,17±0,24	-	-	-	-	-	-
***L. monocytogenes* ATCC 19111**	30,50±0,41	37,67±0,47	20,17±0,24	33,50±0,41	27,67±0,47	18,33±0,47	-	-	-	-	10.17±0.22	10.17±0.44
***B. cereus* ATCC 14579**	25,17±0,24	28,50±0,41	15,17±0,24	26,33±0,47	17,17±0,24	12,50±0,41	-	-	-	-	-	-

d_{well}=6 mm

The biomass and the cell-free supernatant obtained from the cultivation of *Heyndrickxia coagulans* M on medium A exhibited strong antibacterial activity against the rest of the tested pathogens, with inhibition zones ranging from 10 mm to 37 mm. When the strain was cultivated in the medium B a less pronounced activity of the biomass and the cell-free supernatant against *Salmonella enterica* subsp. *enterica* serotype Abony was observed, with inhibition zones of 9 mm. For all other pathogens, the *H. coagulans* M strain showed high activity with inhibition zones of 13 mm to 33 mm depending on the tested pathogen. The biomass of the strain obtained from its cultivation in medium C also showed weak antimicrobial activity against *Salmonella enterica* subsp. *enterica* serotype Abony, which was confirmed by the small diameter of the inhibition zone – 8 mm. Against all other pathogens, the studied strain showed high antimicrobial activity with inhibition zones ranging from 13 mm to 32 mm. The cell-free supernatant of *H. coagulans* M, obtained by culturing it in medium C, did not show antimicrobial activity against both *Salmonella enterica* subsp. *enterica* strains and exhibited weak activity against *Escherichia coli* and *Pseudomonas aeruginosa* with inhibition zones of 9 mm. For the remaining pathogens, the cell-free supernatant showed highly pronounced antimicrobial activity, with inhibition zones ranging from 12 to 18 mm for the different pathogens.

Other authors also report on the antimicrobial activity of *Bacillus coagulans* (*Heyndrickxia coagulans*). Mazhar *et al*. (2024) found that novel strain exhibited activity against multiple fungal and bacterial oral, gastrointestinal, skin and UTI pathogens, including *S. enteritidis, E. coli* and *S. aureus,* which were also inhibited by our newly isolated strain *H. coagulans* M. In another study 31 *B. coagulans* strains were effective against *B. cereus* and *S. aureus* (Kim *et al*., 2020). Abdhul *et al* (2015) demonstrated that *Bacillus coagulans* BDU3 was active against the pathogens *Staphylococcus aureus, Enterococcus* sp. and *Bacillus cereus*.

The results presented in Table 6 show that the second strain – *Heyndrickxia coagulans* BJ exhibited much weaker antimicrobial activity, which was strongly influenced by the composition of the nutrient medium. The lowest activity was observed after cultivation in the medium A. The biomass and the cell-free supernatant demonstrated antifungal activity against *A. niger* and *F. moniliforme* with the inhibition zone diameters being in the range of 9 mm to 10 mm. Against *A. flavus* antifungal activity was observed only for the biomass with an inhibition zone of 9 mm. *Heyndrickxia coagulans* BJ did not exhibit activity against *Candida utilis*, *Saccharomyces cerevisiae* and *Penicillium chrysogenum*. The cultivation in the other two fermentation media led to activity against *A.niger, A.flavus, F.moniliforme* and *P. chrysogenum* with antifungal activity exhibited by both the biomass and the supernatant with inhibition zones in the range of 9 mm to 15 mm. This could be explained by the higher protein content in these media, which is a building block of substances with antimicrobial activity.

As for the antibacterial activity of *Heyndrickxia coagulans* BJ, the strain exhibited almost no effect on the pathogenic microorganisms. It was absent against all test microorganisms when the strain was cultivated in a medium with molasses. In a medium with malt, the strain demonstrated activity only against *S. aureus* and only by the biomass with an inhibition zone of 13 mm. When cultivating *Heyndrickxia coagulans* BJ in medium C, antibacterial activity of the biomass and supernatant was observed only against *L. monocytogenes* with inhibition zones of 10 mm.

According to Ostad *et al*. (2024), no antimicrobial activity of the *B. coagulans* supernatant was detected when applying the agar well diffusion method, but MIC results showed that different concentrations of *B. coagulans* supernatant significantly inhibited the growth of *E. coli, S. typhi, S. flexneri* and *B. cereus*. It is possible that the antibacterial substance produced by our strain did not diffuse well in the agar plate, or that its

concentration was too low to be detected with this method.

4. Conclusions

The present study identified and characterised two bacterial isolates as *Heyndrickxia coagulans.* The two strains exhibited significant antioxidant activity. It was demonstrated that *H. coagulans* M had highly pronounced antibacterial and antifungal activity. It was established that *H. coagulans* M demonstrated higher antimicrobial activity when growing in a medium with molasses, followed by a medium with malt and meat extract medium. Further investigation into the nature of the antimicrobial substances and their safety for humans and animals would allow for the inclusion of the novel strains into probiotic supplements.

5. References

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