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Identification and partial characterization of proteolytic activity of *Enterococcus faecalis* relevant to their application in the dairy industry

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Bacteria of the *Enterococcus* genus represent a group of lactic acid bacteria (LAB). They occur ubiquitous in many traditional fermented foods, especially in artisanal cheeses, playing a beneficial role in the development of cheese flavor. Several enterococcal strains are successfully used as pharmaceutical probiotics and some of them are able to produce bacteriocin and bioactive peptides. However, some *Enterococcus* strains can cause nosocomial infections, including endocarditis, urinary tract infections and bacteremia. Therefore, the questions on their safe use in foods are still valid. The main goals of the study were to investigate a proteolytic potential and to identify key enzymes of the proteolytic system in *Enterococcus faecalis* isolated from artisan Polish cheeses. An extracellular-secreted (E) and a cell envelope proteinase (CEP) were isolated and the enzymes' activities depending on bacterial growth phase were evaluated. CEP displayed a higher protease activity than E, and a CEP fraction has been purified 70-fold by a method that included precipitation, diafiltration and gel filtration chromatography. The molecular mass of the enzyme has been estimated by SDS-PAGE to be ~25 kDa. The maximum enzyme activity of the proteinase has been observed at pH 6.9 and 37°C. The enzyme was able to hydrolyze: casein, bovine serum albumin, α-lactalbumin, β-lactoglobulin, but not Leu-pNa. The results of zymography, SDS- PAGE and LC-MS-MS/MS data had allowed us to identify key enzymes of the proteolytic system of *E. faecalis* as coccolysin and glutamyl endopeptidase. To asses microbiological safety of the tested strain, evaluation of virulence factors presence and antibiotic susceptibility was also conducted.

Key words: *Enterococcus faecalis*, proteolytic activity, coccolysin

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Abbreviations: BCA, bicinchoninic acid; CEP, cell envelope proteinase; DH, degree of hydrolysis; eq, equivalent; E, extracellular-secreted protease; LAB, lactic acid bacteria; Leu-pNa, L-leucine-pNa; LC-MS-MS/MS, liquid chromatography–tandem mass spectrometry; OD, optical density; PCR, polymerase chain reaction; pNa, p-nitroaniline; OPA, o-phthalaldehyde; RSM, response surface methodology; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; WPC, whey protein concentrate

INTRODUCTION

Bacteria of the *Enterococcus* genus are a part of harmless commensal species of the human gastrointestinal tract and are also widespread in the dairy fermented foods. They belong to a group of lactic acid bacteria (LAB). However, the presence of *E. faecalis* in food has been long considered to be a result of unhygienic conditions during production. Whereas, Mundt (1986) had shown that the occurrence of *E. faecalis* in many food products is not always related to direct fecal contamination. Moreover, in the Commission Regulation (EC) No 1441/2007 of 5 December 2007 on microbiological criteria for foodstuffs, a maximum level for the presence of coliforms and *Escherichia coli* was set, as indicators of hygiene, but there was no limit for the enterococci and the presence of enterococci in many food products, especially in cheeses where it is often considered as beneficial. Recent studies indicated that enterococci share interesting biotechnological traits, such as bioactive peptides and bacteriocin production (Foulquie´ Moreno *et al.*, 2006; Hirota *et al.*, 2007; Korhonen & Pihlanto, 2006). Several strains of *E. faecium* (e.g., *E. faecium* SF68®) and *E. faecalis* (e.g., Symbioflor1®) are also used in microbiological therapy, aimed at restoring the balance of the composition of the host intestinal microflora and in the auxiliary treatment of diarrhea (Franz *et al.*, 2011). Surveillance and well established scientific evidence support safety of these strains for both, human and animal applications (Allen *et al.*, 2010).

The positive role of enterococci in the processing of some dairy products is associated with their proteolytic activity, among others (Wilkinson *et al.*, 1994; Giraffa, 2003; Gútiez *et al.*, 2013). Proteolytic system of LAB is composed of extracellular-secreted and surface associated proteinase, peptide transporters and various intracellular peptidases (Law & Haandrikman, 1997; Liu *et al.,* 2010). It plays an important role in generating necessary peptides and amino acids for bacterial growth. Moreover, the secreted and the surface enzymes of the starter or non-starter bacteria are recognized as the molecules which play a key role in the dairy industry because thanks to them the fermented products gain unique organoleptic properties (Broadbent & Steele, 2007; González *et al.*, 2010; Hernández-Ledesma *et al.*, 2011). However, despite the fact that enterococci were isolated from many traditional cheeses manufactured in Spain, Greece, Italy and Poland, previous studies on LAB proteolytic enzymes were mainly restricted to the genera of *Lactobacillus* and *Lactococcus* (Pritchard & Coolbear, 1993; Reid *et al.*, 1994). There are only a few studies regarding the proteolytic system of enterococci in comparison with the other LAB species (González *et al.*, 2010; Pessione *et al.*, 2012). Perhaps the lack of de-

tailed information on the proteolytic enzymes produced by non-starter LAB (including enterococci), may be the reason for the difficulties in transfer of the production of traditional cheeses to the large-scale manufacturing.

On the other hand, enterococci are often described as a biological threat. *E. faecalis* has been mainly implicated as the causal agents of 10% of total nosocomial infections and poses a significant treatment challenge (Kaye, 1982; Murray, 1990; Del Papa *et al.*, 2007). Many studies on enterococcal pathogenicity have focused on investigation of virulence factors in the enterococcal genome which include: extracellular surface proteins, aggregation substance, gelatinase and extracellular superoxide and multidrug resistance (Foulquié Moreno *et al.*, 2006; Lepage *et al.*, 2006; Solheim *et al.*, 2009; Gútiez, 2013). The results have shown that several of the virulence factors are detected in strains of clinical, as well as food origin (Cariolato *et al.*, 2008; Chajęcka-Wierzchowska *et al.,* 2017; Medeiros *et al.*, 2014). However, a higher incidence of some of the virulence determinants was found in the clinical enterococci isolates.

The goals of this research were to investigate the proteolytic activity of *E. faecalis* isolated from artisan Polish cheese and to identify and characterize key enzymes of its proteolytic system. To evaluate microbiological safety of the tested strain, antibiotic susceptibility was determined and the genome was tested for the presence of selected virulence factors.

MATERIALS AND METHODS

Materials. Skim milk powder was purchased from Laktopol (Poland); whey protein concentrate (WPC-80) from Spomlek (Poland); Unstained Protein Molecular Weight Marker from Fermentas (Lithuania); amido black 10B and MRS-broth from Merck Millipore (Darmstadt, Germany); Genomic Mini AX Bacteria Kit and Start-Warm 2 × PCR Master Mix from A&A Biotechnology (Poland); Antimicrobial Susceptibility test discs from Oxoid; Mueller-Hinton agar from Biocorp (Poland). Azocasein, L-leucine-pNa (Leu-pNa), o-phthalaldehyde (OPA), bicinchoninic acid (BCA) kit, lysozyme and Coomassie Brilliant Blue R-250 were purchased from Sigma Aldrich (Poland).

All other chemicals used were of analytical grade and were purchased from POCH (Poland).

Microorganism and growth conditions. *Enterococcus faecalis* 2/28 used in this study was obtained from the culture collection of the Department of Biotechnology and Food Microbiology, Poznań University of Life Sciences (Poland). This strain was recovered from artisan cheeses produced in the Podhale region (Poland). *E. faecalis* was cultured in the MRS broth at 37ºC and stored frozen at –20ºC in the MRS broth supplemented with 20% (w/v) glycerol.

The strain was grown in reconstituted (11%, w/v) skim milk powder. The skim milk was first pasteurized at 90ºC for 20 min and then inoculated. The inoculated milk (2%, v/v) was incubated at 37ºC for 48 h under constant stirring.

Biosynthesis and isolation of bacterial proteases. *E. faecalis* cell fractions were prepared using a modified Requena and others (Requena e*t al.*, 1993) protocol. After 3–48 h of milk fermentation, conducted under conditions as cited above, 100 mL of whey was collected and centrifuged (4 000 × *g*, 10 min, 4°C). The cell free supernatant was stored at –80°C in order to examine the extracellular – secreted protease (E) activity. The remainder of the fermented milk was adjusted to pH 7.0 with 1 M NaOH, and cleared by adding 10% trisodium citrate. The cells ($OD_{600} \approx 1.75$) were harvested by centrifugation (4 000 × *g*, 20 min, 4ºC) and the pellet was washed with 0.05 M Tris-HCl pH 7.0 at 4ºC. To release the cell envelope proteinase (CEP), the cells from 100 mL fermented milk were resuspended in 2 mL of 0.05 M Tris-HCl pH 7.5 to obtain cell density $OD_{600} \approx 24$, where 1 of OD_{600} corresponds to 2 mg of dry weight of biomass. The cell suspension was incubated at 32ºC for 1 h under constant stirring and then centrifuged (4 000 × *g*, 20 min, 4ºC). The supernatant, designated as crude CEP, was stored at –80ºC.

The cell growth in skim milk was evaluated by measuring cell density (OD_{600}) of clarified samples. Cell clarification was performed by an EDTA-borate treatment (Christensen & Steele, 2003).

The protein concentration in the crude enzyme was determined by the bicinchoninic acid (BCA) protein assay, using bovine serum albumin (BSA) as a standard.

Protease enzyme activity. Enzyme activity was measured by a modified method described by Harding *et al.* (1949). The assay mixture contained 1 mL of 2.5% (w/v) azocasein, 0.6 mL of 50 mM Tris-HCl pH 7.5 and 0.4 mL of enzyme. After 60 min incubation at 37ºC under constant stirring (600 rpm), 1 mL of sample was collected and added to 4 mL of 5% (v/v) TCA. Next, the sample was filtered with a 0.45 μm pore size filter (Millipore). 1 mL of the filtrate was mixed with 3 mL of 0.5 M NaOH. Release of the azo dye was detected by measuring absorbance at 440 nm. Endopeptidase activity was calculated with the following equation:

$$\Delta A = A_{\text{sample}} - A_{\text{blank}}$$

where:

A_{sample} – absorbance of the test sample
A_{blank} – absorbance of blank, without enzyme.

One unit [1U] of endopeptidase activity was defined as the amount of enzyme that caused an increase of 0.01 at A_{440} under the assay conditions.

Zymography analysis. Identification of enzymes from *E. faecalis* was carried out by native gel electrophoresis using a 10% resolving gel copolymerized with 2.5% (w/v) azocasein. Crude enzymes were concentrated by ultrafiltration with Amicon Ultra-15 centrifugal filter with a nominal molecular weight of 10 kDa. The fraction of molecular weight ≥10 kDa was applied on the gel and electrophoresis was performed at a constant voltage of 100 V per gel at 4ºC. After electrophoresis, the gel was incubated in zymography-developing buffer for 18 h at 37°C. The gel was stained by using 0.5% solution of amido black in methanol–glacial acetic acid–water (5:1:4). Destaining was carried out using an aqueous solution of 30% methanol and 10% acetic acid. Protein bands with protease activity were revealed as clear zones on a dark background.

Partial purification and characterization of *E. faecalis* CEP

Precipitation. Crude CEP was subjected to 14, 30 and 55% saturated ammonium sulfate precipitation at 4ºC. After each step, the solution was stirred for 25 min and the precipitate collected by centrifugation (4 000 × *g*, 20 min, 4ºC). The pellets were suspended in 50 mM Tris-HCl pH 7.5. The enzyme activity and the protein concentration were monitored at each step of purification as mentioned above.

Diafiltration. The 30–55% precipitate fraction (the highest activity) was dialysed against distilled water using the Amicon Ultra-15 centrifugal filter (10 kDa).

Gel-filtration chromatography. The dialysate fraction precipitated with 30% ammonium sulfate was subjected to gel chromatography with ÄKTA Explorer 100 Air (Amersham Pharmacia, Sweden). The samples were applied to a HiLoad 26/60 Superdex 75 column and eluted with an isocratic system using a 50 mM phosphate buffer pH 7.12 containing 0.15 M NaCl, at a flow rate of 1.0 mL/min. The injection volume of the sample was 11 mL. The wavelength of the UV/Vis detector was set at 280 nm. The fractions were pooled and concentrated using the Amicon Ultra-15 centrifugal filter (with a nominal molecular weight of 10 kDa) for measurement of proteolytic activity.

Effect of temperature and pH on the proteases activity. D-optimal design of response surface methodology (RSM) was used to determine optimum conditions (temperature and pH) for protease activity. Design-Expert 9.0 (Stat-Easa, Inc., USA) was used for the assay design and statistical analysis. Effects of pH (variable code: X_1) and temperature (variable code: X_2) on the CEP protease activity were investigated at five different levels, coded as –1, –0.5, 0, +0.5 and +1. The design consisted of two factors (X_1, X_2) at five equidistant levels (37, 41, 45, 49 and 53 °C) for X_1; 6.0, 6.5, 7.0, 7.5 and 8.0 for X_2. The level chosen was based on results of single-factor experiments.

Enzyme activity (variable code: Y_1) was measured during the D-optimal experiment using 4% (w/v) whey protein concentrate (WPC-80) solution as substrate for 8 h. WPC-80 solutions were prepared in appropriate buffers (0.1 M phosphate buffer in the range of pH 6.0–7.5 and 0.1 M Tris-HCl for pH 8.0) and then heated for 30 min at 65°C (Costa *et al*., 2007). Samples were incubated under assay conditions and then the degree of proteolysis was measured by the OPA method. 15 μL of samples were added to 1 mL of the OPA reagent and after 2 min incubation at ambient temperature, the absorbance was measured at 340 nm. The result was expressed as the difference in absorbance between the hydrolysed WPC-80 and control (unhydrolysed WPC-80).

Enzyme specificity. Endopeptidase activity was measured in terms of azocasein as a substrate. Samples were examined by the method described under "**Protease enzyme activity**" chapter.

Samples for aminopeptidase assay contained 50 μL of (6 mg/mL) L-leucine-pNa, 1.8 mL of 50 mM Tris-HCl pH 7.5 and 0.2 mL enzyme. Samples were incubated at 37ºC for 90 min under constant stirring. The release of *p*-nitroaniline (pNa) was detected by absorbance at 410 nm. One unit [1 U] of aminopeptidase activity was defined as the amount of enzyme required to liberate 1 nM of pNa per 1 min under the assay conditions. The amount of relesed pNa was determined by calibration curve prepared using standard concentrations of pNa. Specific activity was expressed as units per mg protein of the crude enzyme.

The ability to hydrolyse whey proteins was measured in terms of 4% (w/v) WPC-80 solutions released by dissolving in 0.1 M phosphate buffer pH 7.0 and then heated for 30 min at 65°C (Costa *et al*., 2007). The WPC-80 hydrolysis was carried out under optimal pH and temperature for CEP activity with the substrate *vs*. enzyme ratio of 5:1, at 37°C for 24 h. Hydrolysis was stopped by heating in a boiling water bath for 5 min. Samples were centrifuged (4 500×*g*, 10 min, 4°C) and supernatants were stored at –20°C for further analysis. The degree of hydrolysis (DH) was measured using the OPA method and the DH percentage was calculated with the following equation (Spellman *et al*., 2003):

$$DH\ (\%) = \frac{\Delta A \times 1.934 \times d}{c}$$

where:

ΔA is A_{340} of the test sample – A_{340} unhydrolysed sample
d – dilution factor
c – protein concentration (mg/mL).

Additionally, the assessment of whey proteins degradation during enzymatic hydrolysis was monitored by electrophoresis. For this purpose, supernatants were lyophilized and stored at –20°C. Before applying on the gel, samples were dissolved in 0.5 M Tris-HCl buffer pH 6.8. SDS-PAGE was performed by the method described below using 15% gel. Degradation of whey proteins was observed as the reduction in the band intensity during hydrolysis with respect to the original intensity of unhydrolysed proteins.

Determination of molecular mass. Molecular mass of the protease was evaluated by SDS-PAGE using 12% gel, according to standard procedure (Laemmli, 1970). The gel was stained with 0.1% solution of Coomassie Brilliant Blue R-250 in methanol–glacial acetic acid–water (5:2:5). Distaining was carried out using an aqueous solution of 30% methanol and 10% acetic acid. Unstained Protein Molecular Weight Marker was used as molecular weight standard.

Identification of proteases by LC-MS-MS/MS. The E and CEP of *E. faecalis* were analyzed by liquid chromatography coupled to the mass spectrometer (LC-MS-MS/MS) in the Laboratory of Mass Spectrometry, Institute of Biochemistry and Biophysics, Polish Academy of Sciences (Warsaw, Poland).

Samples were concentrated and desalted on a RP-C18 pre-column (Waters), and further peptide separation was achieved on a nano-Ultra Performance Liquid Chromatography (UPLC) RP-C18 column (Waters, BEH130 C18 column, 75 μm i.d., 250 mm long) of a nanoACQUITY UPLC system, using a 45 min linear acetonitrile gradient. Column outlet was directly coupled to the Electrospray ionization (ESI) ion source of the Orbitrap Velos type mass spectrometer (Thermo), working in the regime of data dependent MS to MS/MS switch with HCD type peptide fragmentation. An electrospray voltage of 1.5 kV was used. Raw data files were pre-processed with Mascot Distiller software (version 2.4.2.0, MatrixScience). The obtained peptide masses and fragmentation spectra were matched to the MEROPS peptide database using the Mascot search engine (Mascot Daemon v. 2.4.0, Mascot Server v. 2.4.1, MatrixScience).

Protein identification was performed using the Mascot search engine (MatrixScience), with the probability based algorithm. The expected value threshold of 0.05 was used for analysis.

Detection of potential virulence factors

DNA isolation. Total bacterial DNA was extracted using Genomic Mini AX Bacteria Kit (A&A Biotechnology, Gdańsk, Poland). Briefly, bacterial pellet was resuspended in 100 μL of Tris buffer. After initial incubation in 50 mg/mL lysozyme for 1 h at 37°C, 200 μL of Total Lysis buffer and 20 μL of Proteinase K were added. Cell lysis was done at 37°C for 20 min, and then the sample was transferred to 70°C and incubated for 5 min. Cellular debris was collected by centrifugation for 3 min at 10000–15000 rpm. Supernatant was transferred into the minicolumn, which was washed twice with Wash Solution A1. DNA was eluted using 100 μL of Tris buffer (preheated to 75°C). The quality of the preparations was assessed spectrophotometrically with NanoDrop ND-1000 (NanoDrop Technologies, Inc., USA).

PCR amplification of selected genes. The presence of genes encoding selected virulence factors (*cylA, esp, asa1, hyl, gelE*) in the genome of *E. faecalis* was assessed by multiplex PCR according to the methods of Vankerckhoven and others (Vankerckhoven *et al.*, 2004). The DNA from *E. faecalis* MMH594.1 was used as a reference sample (positive control). PCR was performed in total volume of 10 uL containing 5 uL StartWarm 2 × PCR Master Mix with additional $MgCl_2$ (2.5 mM), 25 ng DNA template, 0.1 µM concentration of each primer specific for *asa1*, *gelE* and *hyl*; and 0.2 µM concentration of each primer specific for *cylA* and *esp*. PCR was carried out with the T Gradient Thermocycler (Biometra, Germany). After initial activation step at 95°C for 10 min, the hot start polymerase was activated. The amplification of selected genes consisted of 30 cycles of 1 min denaturation step at 94°C, 1 min annealing step at 56°C, and 1 min extension at 72°C. Negative controls containing no DNA template were included in parallel. 10 µL samples of PCR product were analyzed by electrophoresis in 2.0% (w/v) agarose in 1 × TBE buffer (89 mM Tris-borate; 2 mM EDTA, pH 8.3) and were subsequently visualized by UV illumination after ethidium bromide staining.

Sensitivity to antibiotics. Antibiotic susceptibility test of *E. faecalis* was carried out with the following antibiotics: penicillin 10 IU, ampicillin 10 mg, gentamicin 30 ug, streptomycin 10 ug, vancomycin 30 mg, imipenem 10 mg, erythromycin 15 mg, tetracycline 30 mg, chloramphenicol 30 µg and rifampicin 5 µg, by the disk diffusion method on Mueller-Hinton agar plates. Classification of *E. faecalis* susceptibility was made according to the inhibition zone diameter, based on The National Reference Centre for Susceptibility Testing (Hryniewicz *et al.,* 2012).

Statistical analysis. All of the experiments were made in triplicate and data was expressed as means ± standard deviation (S.D.).

RESULTS AND DISCUSSION

The selection of *E. faecalis* 2/28 for the source of proteolytic enzymes was due to our previous research, which has shown that a lot of LAB strains, originated from artisan Polish cheeses from raw milk produced in the Podhale region, have the proteolytic ability (unpublished data), however *E. faecalis* 2/28 displayed the most efficient proteolytic activity and it was chosen for further isolation of bacterial proteases.

Biosynthesis of bacterial proteases from *E. faecalis*

The LAB proteolytic system consists of extracellular, cell envelope and intracellular enzymes, belonging to proteinases and peptidases, respectively (Law & Mulholland, 1995; Meijer *et al.*, 1996; Law & Haandrikman, 1997). In this study, the extracellular-secreted (E) and the cell envelope proteases (CEP) were isolated because of their evaluation as the most important enzymes in food production. Taking into account that studies on the effect of bacterial growth phase on proteases activity level are ambiguous (Exterkate, 1985; Laan *et al.*, 1993; Meijer *et al.*, 1996; Pereira *et al.*, 2001), the protease activity of E and CEP and its dependence on bacterial growth phase was evaluated. As shown in Fig. 1, the highest protease activity of E was detected during the exponential growth phase (14.0 ± 0.20 U). The maximum activity of CEP was at the initial stationary phase, at approximately 25 h of milk fermentation (17.1 ± 0.42 U). These results

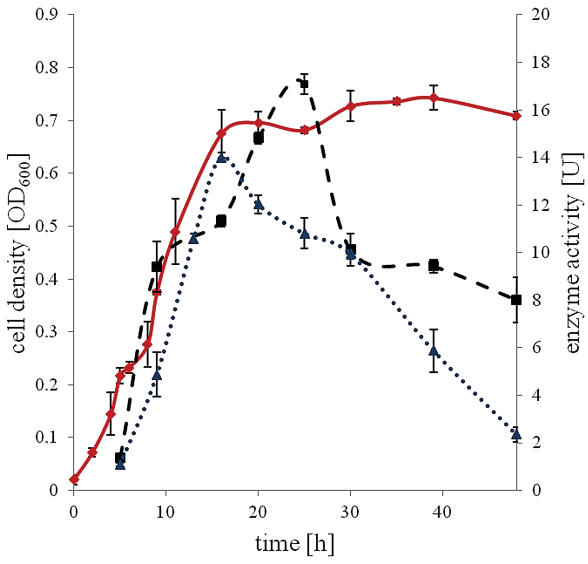

Figure 1. Biosynthesis of extracellular and cell envelope proteinase produced by *Enterococcus faecalis* during milk fermentation
(—) Cell density [OD_{600}]; (· · ·) extracellular enzyme [U]; (– – –) cell envelope proteinase [U]. Assay of enzymatic activity was measured by azocasein. Cell growth during milk fermentation was performed by an EDTA-borate treatment (Christensen & Steele, 2003) and was then evaluated by measuring cell density (OD_{600}).

showed that the specific activity of CEP (20.5 U/mg) was significantly higher than E (1.5 U/mg) and it was observed for a longer period during cell growth. The theory of Thomas and Pritchard (Thomas & Pritchard, 1987) could explain the high activity of enzymes associated with the cell wall. These researchers had stated that this enzyme fraction plays a key role in LAB metabolism, initiating the degradation of milk protein into oligopeptides which are subsequently hydrolyzed to amino acids, i.e. necessary factors for bacterial growth (Meijer *et al.*, 1996).

Zymography analysis

In order to detected the protease in CEP and the E crude fractions, concentrated fractions of ≥ 10 kDa in molecular weight proteins were subjected to zymography analysis. Casein zymography was performed in native polyacrylamide gels containing azocasein (2.5%) as a copolymerized substrate. The zymography analysis indicated that the tested protein fractions (CEP and E) contain

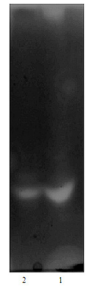

Figure 2. Zymogram of cell envelope proteinase (CEP) and extracellular-secreted protease (E)
Zymography was performed with native polyacrylamide gels containing azocasein (2.5%), stained with amido black. Clear colorless zones on a dark background gel indicated protease activity. Lane 1, cell envelope proteinase (CEP); lane 2, extracellular-secreted protease (E).

Table 1. Purification of cell envelope proteinase (CEP) of *Enterococcus faecalis*
Proteolytic activity was measured by azocasein. The protein concentration was determined by the bicinchoninic acid (BCA) protein assay.

Purification step	Volume [mL]	Total protein [mg]	Total activity [U]	Specific activity [U/mg]	Purification (fold)
Crude enzyme	172	715	14693.1	20.5	1.0
Ammonium sulphate precipitation	22.19	115.4	3292.4	28.5	1.4
Diafiltration	3.6	24.8	1614.6	65.1	3.2
Superdex 200 + UF (30 kDa)	1.06	0.22	343.8	1448.8	70.7

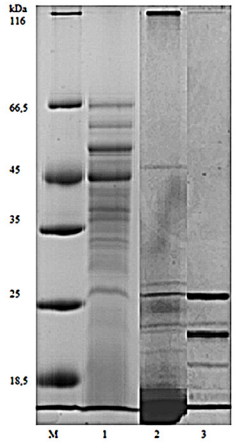

Figure 3. SDS-PAGE for the cell envelop proteinase (CEP) of *Enterococcus faecalis*
SDS–PAGE analysis was performed at different purification steps using 12% polyacrylamide gel, stained with Coomassie Blue. Lane 1, crude CEP; lane 2, CEP after precipitation and diafiltration; lane 3, CEP after gel filtration chromatography (P1 fraction); lane M, molecular weight marker (Unstained Protein Molecular Weight Marker).

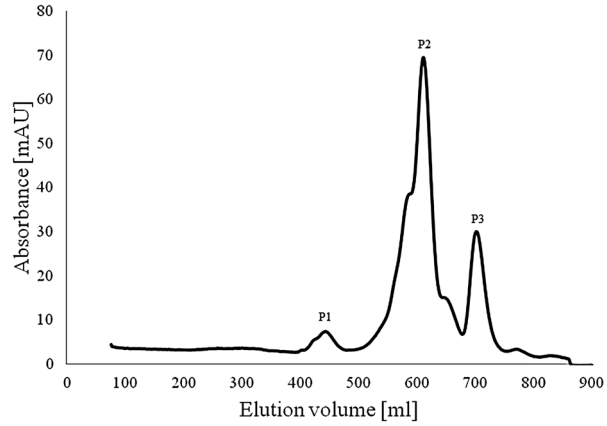

Figure 4. Gel filtration of the 30–55% precipitate fraction
Gel filtration chromatography was performed on HiLoad 26/60 Superdex 75 column. P1, P2, P3 – separated fractions.

active proteases, which was observed as a clear colorless zones on a dark background (Fig. 2).

Partial purification and characterization of *E. faecalis* CEP

Based on preliminary results of the enzyme activity, the CEP fraction was used for further analysis. The *E. faecalis* CEP was partially purified using 3 steps: precipitation, diafiltration and gel-filtration chromatography. A purification scheme, which resulted in a 70-fold puri-

Table 2. Results of D-optimal design of RSM
Conditions of experiments (X_1: pH, X_2:temperature) were determined by D-optimal design of RSM. Enzyme activity was tested by 4% (w/v) whey protein concentrate (WPC-80) solution.

Variant of the experiment	Variable		
	X_1: pH	X_2:Temperature [°C]	Y_1: Enzyme activity
1	7.5	49	0.099707
2	6	53	0.032107
3	7	53	0.069107
4	8	37	0.205907
5	7	41	0.340607
6	6.5	49	0.152607
7	8	45	0.159107
8	7	45	0.310807
9	8	37	0.332707
10	6	45	0.173007
11	6	37	0.334607
12	6	53	0.003407
13	7	45	0.193707
14	7	37	0.344107
15	6	37	0.275707
16	8	53	0.034007

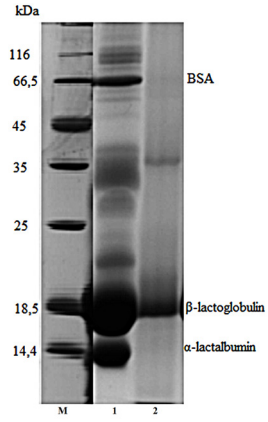

Figure 5. SDS-PAGE of WPC-80 hydrolysate obtained after treating WPC-80 with CEP of *Enterococcus faecalis*
SDS-PAGE analysis was performed with 15% polyacrylamide gel, stained with Coomassie Blue. Lane M, molecular weight marker (Unstained Protein Molecular Weight Marker; 14.4–116 kDa); lane 1, native WPC-80; lane 2, WPC-80 hydrolyzed by CEP for 24 h.

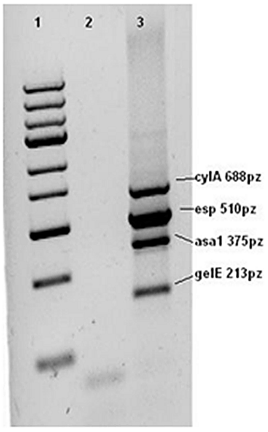

Figure 6. Multiplex PCR of *Enterococcus faecalis*
Lane 1, molecular weight marker (GeneRuler Express DNA Ladder, bands: 5000, 3000, 2000, 1500, 1000, 750, 500, 300, 100,); lane 2, negative control (no DNA added); lane 3, *Enterococcus faecalis*.

fication, was developed. The results are summarized in Table 1.

CEP were analyzed at different purification steps by SDS-PAGE (Fig. 3). After the final purification step, four protein bands were detected in the pooled active P1 fraction (Fig. 4) with a molecular mass which ranged from 25 to 18.5 kDa. A similar result was reported by Garcia de Fernando and others (Garcia de Fernando *et al.*, 1991), who investigated the extracellular protease produced by *E. faecalis* subsp. *liquefaciens,* and demonstrated that the molecular mass of the enzyme was ~ 26 kDa by SDS PAGE and ~30 kDa by Sephadex gel filtration.

Effect of pH and temperature

D-optimal design of RSM was used to determine the optimal conditions for enzyme activity. Table 2 shows the results of experiments. The tested crude CEP showed maximum activity at 37°C, while optimum pH was 6.9.

Enzyme specificity

In this study, enzyme specificity of CEP was evaluated by measurement of endopeptidase and aminopeptidase activity by using azocasein and L-leucine-pNa as substrates, respectively. CEP was able to hydrolyze azocasein, but did not show activity against Leu-pNa. The endopeptidase activity level was 17.1 U and the aminopeptidase activity level was not observed.

The ability of proteases from *E. faecalis 2/28* to hydrolyse whey proteins was also evaluated. It was shown that CEP have the ability to hydrolyze WPC-80. The degree of hydrolysis after 24 h equaled 20.3%. In order to evaluate the degradation of whey proteins during enzymatic hydrolysis, electrophoresis was carried out. SDS-PAGE showed distribution of whey protein during enzymatic hydrolysis. As shown by electropherogram (Fig. 5), after 24 h hydrolysis, serum albumin and α-lactalbumin were degraded altogether while the degradation of β-lactoglobulin was significant but not full. This is in line with results obtained by Garcia de Fernando and others (Garcia de Fernando *et al.*, 1991), who demonstrated that the extracellular protease produced by *E. faecalis* subsp. *liquefaciens* was able to hydrolyze casein, α-lactoalbumin, β-lactoglobulin and bovine serum albumin.

Whey proteins are considered to be substrates resistant to enzymatic hydrolysis (Babij *et al.*, 2013; Spellman *et al.*, 2003). The use of commercial enzymes for their proteolysis often does not give satisfactory results. The results of the study present here indicate that proteases from *E. faecalis 2/28* exhibit proteolytic activity towards whey proteins, which could imply their potential application in the dairy technology and biotechnology, e.g. biopeptide production.

Table 3. Sensitivity of *Enterococcus faecalis* to selected antibiotics
Sensitivity to antibiotics was performed by the disk diffusion method on Mueller-Hinton agar plates using standard Antimicrobial Susceptibility test discs (Oxoid).

Antibiotic	Dose of antibiotic	Inhibition zone diameter (mm)	Sensitive (+)/ lack of sensitive (–)
Penicillin	10 IU	18	+
Ampicillin	10 µg	14	+
Gentamicin	30 µg	14	+
Streptomycyn	10 µg	–	–
Vancomycin	30 µg	16	+
Imipenem	10 µg	30	+
Erythromycin	15µg	–	–
Tetracycline	30 µg	–	–
Chloramphenicol	30 µg	10	+
Rifampicin	5 µg	14	+

Identification of proteases by LC-MS-MS/MS

Protease expression is strain specific and it is not representative for the whole species of *Enterococcus faecalis* (Pessione *et al.*, 2012). Thus, in order to identify the proteases of the CEP fraction, i.e. the key enzymes for proteolytic system of the tested strain, LC-MS-MS/MS analysis was performed. Based on the results of zymography and SDS PAGE, together with MS/MS data and literature information (Garcia de Fernando *et al.*, 1991; Pei-Show, 2002), it revealed that the key enzymes of proteolytic system of *E. faecalis* 2/28 are coccolysin (gelatinase) and glutamyl endopeptidase.

Gelatinase (GelE) and glutamyl endopeptidase (serine proteinase V8, SprE) are extracellular proteases. Their respective encoding genes, *gelE* and *sprE,* are located in the same operon and are co-transcribed (Pessione *et al.*, 2012). They are known as toxic substances secreted by *Enterococcus* strains which have a destructive effect on the host's tissues. GelE has been also reported to contribute to bacterial adherence and biofilm formation (Anderson *et al.*, 2016; Chajęcka-Wierzchowska *et al.*, 2017; Franz *et al.*, 2003). On the other hand, there is evidence that proteases from enterococci could find potential application in the processing of some dairy products. According to Giraffa (Giraffa, 2003), the presence of enterococci as non-starter LAB or added as adjunct starter throughout ripening of cheeses, positively affected their taste, aroma, structure and color. This seemed linked to the fact that in the cheeses made with enterococci, total free amino acids, soluble nitrogen, long-chain free fatty acids, volatile free fatty acids and acetoin diacetyl and contents were generally higher, as the result of bacterial enzymatic activity. Recent research also suggested that the gelatinase may have interesting biotechnological traits, e.g. production of bioactive peptides (Gútiez *et al.*, 2013).

Detection of potential virulence factors

Enterococci have a beneficial effect in the dairy food production and a long history of safe use, however, they are characterized by natural resistance to numerous antibiotics. Some *Enterococcus* strains isolated from food also carry virulence factors typical for nosocomial pathogens, such as *ace, asa1, gelE,* and *esp* (Chajęcka-Wierzchowska *et al.*, 2017; Pessione *et al.*, 2012). Nevertheless, there are no legal regulations that would allow to exclude those strains which may cause infections, from the use in the industry.

In view of the above, in this study, the evaluation of *E. faecalis* properties which are relevant in biotechnology was carried out with respect to their potential pathogenicity as well. Taking into account that virulence in the *Enterococcus* genus has been considered as a multifactor process, with participation of several genes and their products (Eaton & Gasson, 2001; Vankerckhoven *et al.*, 2004; Kawalec & Jakubczak, 2006), the genome of *Enterococcus faecalis* was examined for the presence of genes encoding selected virulence factors (*cylA, esp, asa1, gelE, hyl*).

The analysis showed that *E. faecalis* was positive for *cylA*, *esp*, *asa1*, *gelE* genes encoding cytolysin, enterococcal surface protein, aggregation substance and gelatinase (coccolysin), respectively, but the *hyl* gene encoding hyaluronidase was not detected (Fig. 6). Accordingly, the analyzed strain lacks gene for hyaluronidase which is believed to contribute to invasion of the nasopharynx and to pneumonia.

Other research also proved that strains isolated from food may possess virulence determinants (Cariolato *et al.*, 2008; Eaton & Gasson, 2001 and Gútiez *et al.*, 2013; Medeiros *et al.*, 2014). For example, Medeiros and others (Medeiros *et al.*, 2014) compared strains isolated from food and from material of clinical origin and showed that regardless of the source of isolation, the incidence of the gelatinase gene (*gelE*) and the collagen binding protein (*ace*) is at the same level. Cariolato and others (Cariolato *et al.*, 2008) noted that virulence factors were present in both, the dairy and human strains, and had shown that *E. faecalis* strains isolated from dairy products have from 1 to 6 virulence factors in their genome. More importantly, Domann and others (Domann *et al.*, 2007) pointed out that both, *asa1* and *esp,* are equally present in the commercial probiotic strain of *E. faecalis* Symbioflor1. On the other hand, Eaton and Gasson (Eaton & Gasson, 2001) revealed that the frequency of occurrence of virulence determinants is higher among hospital isolates than strains derived from food. In contrast, Anderson and others (Anderson *et al.*, 2015) demonstrated that the food isolates possessed more *asa1* and *esp* genes than the clinical isolates, and also possessed more *esp* and *gelE* genes than the food isolates studied by Eaton and Gasson (Eaton & Gasson, 2001). Anderson and others (Anderson *et al.*, 2015) suggested that a high percentage of these virulence factors is reported for isolates originated from raw milk.

However, the role of some virulence factors, such as aggregation substances, adhesions, gelatinase, or cytolysin in enterococcal pathogenicity is unclear. For example, the gelatinase and the serine proteinase V8 have been described as major virulence factors of the *Enterococcus* genus (Qin *et al.*, 2000; Thurlow *et al.*, 2010). On the other hand, high frequency of *gelE* gen occurrence in the genome of strains isolated from dairy food, may suggest that gelatinase has an important function in the metabolism of these bacteria (Anderson *et al.*, 2015; Medeiros *et al.*, 2014). Moreover, enterococci are also capable to produce bacteriocins, such as cytolysin and enterolysin, with activity against *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium botulinum* or *Vibrio cholerae* and can be used as probiotics (Giraffa, 2003). The presence of the *ace* and *esp* genes encoding adhesins, is seen by many researchers as one of the factors having important roles in the early stages of infection and facilitating colonization of the host (Shankar *et al.*, 2001; Gilmore *et al.*, 2002). In contrast, Archimbaud and others (Archimbaud *et al.*, 2002) found no link between the presence of gelatinase, cytolysin and aggregation substance, and the ability of *E. faecalis* strains to adhere to heart cells in an *in vitro* study.

Sensitivity to antibiotics

Clinical importance of bacteria of the *Enterococcus* genus is directly related to their natural antibiotic resistance, e.g. to cephalosporins, cotrimoxazol or clindamycin (Hryniewicz *et al.*, 2012) and their ability to transfer resistance genes to other microorganisms (Ortigosa *et al.*, 2008), which contributes to the risk of infection. In the light of the above, the susceptibility to antibiotics of the tested strain was determined. The selection of antibiotics for susceptibility testing was in accordance to the recommendation of The National Reference Centre for Susceptibility Testing (Hryniewicz *et al.*, 2012). On the basis of the antibiograms provided, it was shown that the tested strain was susceptible to penicillin, ampicillin and gentamycin, i.e. the first-line antibiotic treatments of infections caused by bacteria of the *Enterococcus* genus. The tested strain was also susceptible to vancomycin, imipenem, chloramphenicol and rifampicin, i.e. medicines that

are intended for treatment of infections caused by multidrug-resistant enterococci. However, the tested strain was resistant to streptomycin, tetracycline, and erythromycin, i.e. antibiotics indicated as add-on therapy of enterococcal infections. The results of the antibiotic susceptibility of *E. faecalis* are presented in Table 3.

The resistance of *E. faecalis* to streptomycin is probably dependent on the antibiotic dose used. The recommended concentration of streptomycin for the treatment of enterococcal infections is 300 μg and in this study a dose of 10 μg was used. However, the sensitivity to vancomycin is of particular clinical importance because vancomycin belongs to the group of glycopeptides considered as the last line of defence for patients who suffer from opportunistic infections.

According to some researchers, the pathogenic potential of enterococci is small as evidenced by their presence as part of the normal microflora of gastrointestinal tract, as well as a long history of safe use as starter cultures and probiotics (Franz *et al*., 2003; Semedo *et al*., 2003). In fact, enterococci from food, which are carriers of virulence genes, very rarely cause disease in healthy people, which seems to confirm this thesis. Nevertheless, it should be remembered that enterococci have different mechanisms of gene transfer, hence there is a risk of transferring antibiotic resistance genes and virulence genes to other microorganisms (Franz *et al*., 2003). These data indicate that assessment of sensitivity of enterococci to drugs should be obligatory in this kind of research.

CONCLUSION

As shown in this study, *E. feacalis* is able to produce active protease which indicates a possibility of its application in the dairy industry, e.g. to support the breakdown and release of bioactive peptides from whey protein. We also demonstrated that the *E. faecalis* strain was positive for *cylA, esp, asa1, gelE* genes. Nevertheless, it should be pointed out that the risk assessment on the basis of virulence factors is a problem because the knowledge regarding the type and combination of virulence factors, which are decisive for pathogenic potential, is limited. To our knowledge, there are no legal regulations on the safety of *E. faecalis* use as a starter cultures or even as a probiotics. Moreover, some of the requirements for probiotics, such as: ability to adhere to cells or produce bacteriocins antagonistic to pathogens, make the same factors responsible for both, the virulence and health benefits, e.g. adhesions.

Furthermore, there is no evidence showing a direct relationship with the consumption of food containing *Enterococcus* strains harboring some virulence factors and diseases, but the use of enterococci for food production gives rise to controversies. Some researchers believe that virulence of enterococci is mild because in most cases these pathogens usually cause infections in patients who are debilitated by other illnesses and undergoing prolonged hospitalization (Kawalec & Jakubczak, 2006; Arias & Murray, 2012).

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