Effect of seafood peptones on biomass and metabolic activity by *Enterococcus faecalis* DM19

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Abbreviations:

SQES : Squid protein hydrolyzed with Esperase

SQAP : Squid protein hydrolyzed with Alkaline protease

SQTR : Squid protein hydrolyzed with Trypsin

SHAP : Shrimp protein hydrolyzed with Alkaline protease

SHNP : Shrimp protein hydrolyzed with Neutral protease

SHEE : Shrimp protein hydrolyzed with endogenous enzymes

FGTR : Fish gelatin hydrolyzed with Trypsin

SHPR : Shrimp protein hydrolyzed with Protamex

Abstract

Eight seafood protein hydrolysates (SPHs) obtained from squid, shrimp and fish gelatin were incorporated as substitutes of peptones in culture media in order to evaluate its effect on survival and metabolic activity (lactic acid, acetic acid and bacteriocins production) of *Enterococcus faecalis* DM19. The substitution of commercial peptones in culture media by either a shrimp hydrolysate prepared with Protamex, or by squid protein hydrolysates prepared with Esperase or Alkaline protease, stimulated *E. faecalis* DM19 growth up to 16%. The incorporation of SPHs, mainly from shrimp, in the culture media significantly increased production of lactic and acetic acids in more than 60%. Furthermore, the media containing SPHs stimulated antimicrobial activity by *E. faecalis* DM19. The inhibitory activity was observed against both Gram-positive and Gram-negative microorganisms, but it was remarkably observed against *Listeria monocytogenes*. SPHs incorporated in culture media render properties of bio-technological interest, which, together with their low price, make them suitable for industrial use.

Keywords: *Enterococcus faecalis* DM19, seafood protein hydrolysates, bacteriocinogenic activity, antimicrobial activity, renewable materials

1. Introduction

The genus *Enterococcus* comprises species considered components of the human and animal intestinal flora (Khan, Flint, & Yu, 2010) and is generally found in large numbers in dairy products and fermented foods. Lactic and acetic acid, diacetyl, and bacteriocins are some of the molecules produced by these microorganisms that present antimicrobial activity (Gilmore, Lebreton, & van Schaik, 2013). Moreover, several *E. faecalis* strains have been investigated as potential probiotics (Franz, Huch, Abriouel, Holzapfel, Gálvez, 2011). Therefore, from the viewpoint of their industrial importance, *E. faecalis* could play a significant role in fermentation and preservation of some foods (Khan, Flint, & Yu, 2010).

Like other lactic acid bacteria (LAB) groups, enterococci can only grow in a rich microbial growth medium which contains all the necessary compounds such as amino acids, peptides, fatty acids, vitamins and nucleic acids required for optimal growth and metabolite production. These compounds are usually provided in the form of complex nitrogen sources such as yeast extract, meat extract, tryptone, peptone or bactopeptone. Those nitrogen sources can reach high prices and be unsuitable for industrial scale production of microorganisms (Zhang & Gresham, 1999). Thus, there is great interest in investigating cheap renewable nitrogenous materials to prepare a low-cost fermentation medium. Several authors (Aspmo, Horn, & Eijsink, 2005; Hujanen & Linko, 1996; Li, Han, Ji, Wang, & Tan, 2010; Yu, Lei, Ren, Pei, & Feng, 2008) studied various nitrogen sources (yeast extract, malt sprout, peptones, grass extract, corn steep liquor, casein hydrolysates, distiller's waste, ammonium phosphates, wheat bran, fish waste hydrolysates and urea) for lactic acid fermentation. The use of

autolysates as a peptone source in bacteriocin production by LAB was also studied (Deraz, El-Fawal, Abd-Ellatif, & Khalil, 2011). Nevertheless, to our best knowledge, there is no information about the stimulation of lactic acid and/or production of bacteriocin by *E. faecalis* in MRS media formulated with seafood (crustacean and cephalopods) protein hydrolysates.

The aim of the present work was to evaluate the effect of different seafood protein hydrolysates as a peptone source on biomass production of *E. faecalis* DM19. The production of organic acids (lactic, acetic and formic acids) and bacteriocins in these culture media were also studied.

2. Material and methods

2.1. Bacterial strain

The *E. faecalis* DM19 was obtained from the Laboratory for Biology of Microorganisms and Biotechnology (University of Oran 1 Ahmed Benbella, Algeria). The strain was originally isolated from raw camel milk collected in the Mauritania area and identified by PCR amplification using *E. faecalis* primers and 16S rDNA sequencing (Vincent, Roy, Mondou, & Dery, 1998). A search for homology of the DNA sequence was performed using the BLAST algorithm available at the National Center for Biotechnology Information (NCBI, USA).

2.2. Preparation of seafood protein hydrolysates

Squid (*Loligo vulgaris*) muscle trimmings, by-products (muscle trimmings, carapace and heads) derived from processing Pacific white shrimp (*Penaeus vannamei*) and Southern pink shrimp (*Penaeus notialis*), and high molecular weight (HMW) dried fish skin gelatine (type A) of cold water fish (Sebastes spp.) were used as raw material to prepare seafood protein hydrolysates. The by-

products were minced and hydrolysed with commercial or endogenous enzymes (Table 1) which were kindly provided by Novozymes (Denmark). Enzymatic reactions were controlled by using a pH-stat. After hydrolysis, the samples were rapidly heated to 90°C for 15 min to inactivate the enzymes. The hydrolysates were then centrifuged at 10000 x g for 20 minutes at 4ºC (Beckman J2-MC, Palo Alto, California, USA). The degree of hydrolysis for each hydrolysate was calculated according to Spellman, McEvoy, O'Cuinn, and FitzGerald (2003). The supernatants were freeze-dried under vacuum and kept at -20°C until their use in the culture media formulation.

2.3. Culture media

Peptone in MRS (Man, Rogosa and Sharpe, Merck KGaA, Darmstadt, Germany) media was substituted by protein hydrolysates (marine peptones) at two different concentrations as depicted in Table 2 (MRS –standard culture media- was considered as control and MRS^{-} -without peptone- as negative control). After adjusting the pH to 7, the media were autoclaved at 121°C for 20 min. Inoculum (1%, v/v) consisting of cellular suspension of an 18 h young culture of *E. faecalis* DM19 in MRS broth medium, was adjusted to an OD (600 nm) of 0.900 (UV-Visible spectrophotometer model UV-1601 from Shimadzu). Then, the broth was centrifuged at 12000 x g (Microspin 24S from Sorvall instruments) for 10 min at 4°C and the sediment was washed twice with phosphate buffer (0.1M, pH 7.0) to remove all traces of the culture medium. The washed cells were re-suspended in sterile distilled water and then adjusted to the initial OD (600nm).They were then used to inoculate modified MRS medium. After 24 hours of incubation at 30°C, an aliquot of the medium was taken to determine bacterial growth. The rest of the medium was centrifuged at 5000 x g

for 20 min at 4°C (Sorvall, model RT 6000B). The supernatant was used for the assessment of glucose, total nitrogen, organic acids (lactic acid, acetic acid, formic acid) and to determine the bacteriocinogenic activity.

2.4. Analytical methods

Cell growth was directly monitored by measuring the optical density of the culture broth samples at 600 nm using spectrophotometer (UV-Visible spectrophotometer model UV-1601 from Shimadzu). The evolution of pH in the culture broth was determined with a pH meter (inoLab, D-82362 Weilheim Germany).

The total nitrogen content was determined using the Dumas combustion method following AOAC 992.15 (1995) in a LECO model FP-2000 (Leco Instruments, Madrid, Spain) protein/nitrogen calibrated with EDTA. Protein content was estimated by multiplying the total nitrogen content by the factor 6.25, and was expressed as grams of protein/100 grams of sample.

Glucose and organic acid content (lactic, acetic, formic, pyruvic and citric acids) were determined by HPAEC-PAD (High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection) using a Metrohm Advanced Compact ion chromatography instrument (867 IC. Metrohm) equipped with an IC-819 conductivity detector, an IC Pump 818 and an IC-837 degasser coupled. Firstly, the supernatants (obtained as previously described) were diluted in ultrapure water and then filtered through a membrane of 0.45 µm pore size. For glucose determination, 20 µL of samples were injected into a Hamilton RC X 30 (250 x 4 mm, 5 µm pore size) column. The samples were eluted from the column over 35 min at a flow of 1 mL/min with an isocratic gradient of 30 mM NaOH with 3 mM AcNa. For organic acids, the identification

and quantification were carried out by injecting 20 µL of the samples into Metrosep Organic Acid (250 x 4 mm, 5 µm pore size) column and the elution was performed with 1 mM perchloric acid and acetone (10 %) as mobile phase over 20 min at a flow of 0.5 mL/min. All the molecules studied were identified by their retention times and quantified based on calibration curves derived from standards. The results were expressed as mg/L of the culture supernatant. It is evident that concerning the accumulation of acetic acid into the culture medium, the initially added sodium acetate concentration (=5.0 g/L) was taken into consideration for the calculations performed. All assays were carried out in triplicate.

2.5. Bacteriocinogenic activity and whole genome sequencing (WGS) of *E. faecalis* DM19

Culture supernatants from the different media tested were filtered through a cellulose acetate sterile filter (0.22 µm) to remove bacterial cells. The extracts obtained were then lyophilized and stored at – 20°C. The samples were re-suspended in distilled water to a concentration of 5 mg/mL for the culture extracts. Finally, the pH of the prepared solutions was adjusted to 7.0 with NaOH (3N).

The antimicrobial activity of the supernatant was tested with the disc diffusion method (Arancibia, Giménez, López-Caballero, Gómez-Guillén, & Montero, 2014). Briefly, sterile paper discs (5 mm diameter, Whatman No. 1) impregnated with 40 µL of the supernatant solutions were placed on the surface of agar plates inoculated with several microorganisms from the Spanish Type Culture Collection (CECT) selected due to their relevance in human health (either lactic acid bacteria or pathogens) or to their role in food spoilage: *Aeromonas*

hydrophila CECT 839T, *Aspergillus niger* CECT 2088, *Bacillus cereus* CECT 148, *Bacillus coagulans* CECT 56, *Bifidobacterium bifidum* DSMZ 20215, *Brochothrix thermosphacta* CECT 847, *Citrobacter freundii* CECT 401, *Clostridium perfringens* CECT 486, *Debaryomyces hansenii* CECT 11364, *Enterococcus faecium* DSM 20477, *Escherichia coli* CECT 515, *Lactobacillus helveticus* DSM 20075, *Listeria monocytogenes* CECT 4032, *Penicillium expansum* DSMZ 62841, *Pseudomonas fluorescens* CECT 4898, *Salmonella choleraesuis* CECT 4300, *Shewanella putrefaciens* CECT 5346T, *Shigella sonnei* CECT 4887, *Staphylococcus aureus* CECT 240, *Vibrio parahaemolyticus* CECT 511T, *Yersinia enterocolitica* CECT 4315. After incubation, the diameter of the inhibition zone (considered antimicrobial activity) was measured with Corel Draw X6 software. Results were expressed as mm of inhibited growth area (magnification ratio 1:10). Each determination was performed in triplicate.

In order to evaluate which type of bacteriocin was present, the genome of *E. faecalis* DM19 was sequenced by whole-genome sequencing (WGS) method (Goodwin, McPherson, & McCombie, 2016). The DNA libraries were sequenced in the Illumina HiSeq platform, using 100bp paired-end sequencing reads. The raw sequence data of the sample was *de novo* assembled using algorithm SPAdes 3.9 (Bankevich et al., 2012). Bacteriocin genes were identified using the bacteriocin mining tool Bagel3 (Van Heel, de Jong, Montalbán-López, Kok, & Kuipers 2013). Each bacteriocin-like gene identified was checked using BLASTX algorithm.

2.6. Statistical analysis

Statistical tests were performed using the SPSS computer program (SPSS Statistical Software, Inc., Chicago, IL, USA). Two-way analysis of variance (ANOVA) was carried out. The difference of means between pairs was resolved by means of confidence intervals using a Tukey-b test at a significance level of 5%.

3. Results and discussion

3.1. *Effect of SPHs on growth*

The substitution of commercial peptone in culture media by hydrolysates from squid (SQTR and SQAP) and prawn (SHPR), especially at two-fold concentration, significantly increased the biomass ($maxOD_{600}$) of *E. faecalis* DM19 (Table 3). In contrast, the growth in media where SHAP or FGTR (one or two-fold) were incorporated was significantly lower when compared with that of commercial MRS. In addition, the terminal pH in *E. faecalis* DM19 cultures was quite low as expected (Table 3). The data show an inverse correlation between biomass production and terminal pH.

Some studies have been carried out to assess the ability of commercial proteolytic enzymes from different resources (plants, animals or microorganisms) to reduce fish protein (whole fish or wastes) into peptides and amino acids, which can be an economical source of peptones for bacterial growth media. In fact, protein hydrolysates from seafood by-products can be possible substitutes for the usual protein hydrolysate-based substrates in microbial cultures (Gao, Hirata, Toorisaka, & Hano, 2006). In this work, seafood peptones showed an activity which was in the same range as the commercial peptone activity or scarcely higher in some cases, suggesting a well-balanced

219 nutrient concentration of these SPHs media. Stein, Svein, and Vincent (2005a)
220 reported that since peptones may differ in buffer capacity and the terminal pH
221 values reached in the *Escherichia coli* cultures were low, the observed growth
222 differences were due to the pH/buffering effect. In the present work, *E. faecalis*
223 showed a different biomass after fermentation in media including SPHs at a
224 similar pH (Table 3). Vázquez, González, and Murado (2004) found similar
225 results when testing the growth of *Roseobacter* sp on MRS supplemented with
226 squid hydrolysate at different concentrations. These authors attributed this
227 phenomenon to the presence of compounds (perhaps lipidic peroxides) with a
228 slight inhibitory effect on the corresponding microorganism. Moreover, the
229 peptidic composition of the SPH added to the MRS media may also affect
230 bacterial growth (Table 3) since the results obtained from MRS media including
231 hydrolysates derived from the same substrate (squid or shrimp) were different
232 ($P \leq 0.05$). In this connection, Stein, Svein, and Vincent (2005b) cultivated LAB
233 on MRS media supplemented with hydrolysates from cod viscera prepared with
234 Alcalase, Papain and endogenous enzymes and found that peptone
235 performance is highly dependent on composition details as peptide length,
236 peptide sequences, and the amount of free amino acids. In the present work, *E.*
237 *faecalis* DM19 exhibited a clear preference for MRS media including one-fold
238 concentration of squid protein hydrolysates prepared with Esperase or Alkaline
239 protease (SQES and SQAP, respectively), or shrimp protein hydrolysate
240 prepared with Protamex (SHPR). However, the incorporation of a shrimp protein
241 hydrolysate prepared with an alkaline protease (SHAP) in the media affected
242 negatively bacterial growth. The amount of SPHs incorporated in MRS media
243 also seems to be important in the biomass production (Table 3). The results

244 may be explained by the fact that certain proteases can release amino acids
245 and peptides suitable for the transport of bacteria and proteolytic systems
246 (Kunji, Mierau, Hagting, Poolman, & Konings, 1996). Moreover, certain
247 hydrolysates as SHAP or FGTR may contain antimicrobial peptides or
248 compounds that negatively affect bacterial growth (Van't Hof, Veerman,
249 Helmerhorst & Amerongen, 2001).

250 The production of organic acids by *E. faecalis* DM19 could also be affected by
251 the composition of the MRS media. If the culture medium stimulates the
252 production of organic acids by the bacteria, these acids could decrease the pH
253 of the media and regulate bacterial growth significantly. This fact could explain
254 why the media prepared with two-fold concentration of SHEE promoted a low
255 production of biomass and a high production of acetic acid. Different
256 investigations have been driven in this way to confirm the inhibitory effect of
257 acetic acid on several microorganisms. Recently, Boguta and Jensen (2014)
258 reported that, for *Pediococcus acidilactici*, 5.3 g/L acetate caused a 4% growth
259 inhibition and showed a synergistic effect when the strain was grown with both
260 acetate and furfural. Therefore, the inhibition phenomenon shown for SHEE
261 was probably due to the high concentration of acetate produced by *E. faecalis*
262 (Table 3).

263 **3.2. *Effect of SPHs on production of organic acids and consumption of***
264 ***glucose and protein***

265 The amount of glucose and protein in the different media were measured before
266 and after the fermentation process (Table 4). Glucose and protein consumptions
267 for bacteria were higher in media with one-fold concentration of SPHs. The

highest glucose consumption was found in media containing SQES. Glucose consumption augmented when a higher amount of SPHs was added in the media, while protein consumption decreased significantly. Protein consumption was minimal in media including SPHs prepared with alkaline protease (SQAP and SHAP).

Acid production depended on the media and on peptone concentration (Table 4). Lactic and acetic acids were the major products of the fermentation process with *E. faecalis* DM19, while formic acid was produced in low quantities. The incorporation of SPH in the media improved notably the production of lactic acid in all samples, and that of acetic acid especially in SHPR-S and SHEE-S samples. Interestingly, the highest production of lactic acid was achieved in media with the mentioned hydrolysates.

As describe before, SHPR is the best supplement for efficient lactic acid production in medium of 1f. Furthermore, the increment for SPH in the media (2f) exerted a positive effect on lactic acid production (Table 4). The quantities produced in SHAP-S and SHPR-S samples were 1.6 times larger compared to those in MRS medium. These differences in production could be due to the marine peptones used in fermentation, since the strain was cultivated under the same experimental conditions. In this regard, lactate production increased in *Streptococcus zooepidemicus* when tryptone was replaced by marine peptones (Vázquez, Montemayor, Fraguas, & Murado, 2009) while the increase in wheat bran concentration promotes lactic acid production by *Lactobacillus rhamnosus* LA-04-1 (Zheng, Lu, Yizhi, Xiaonan, & Tianwei, 2010). *E. faecalis*, like other LAB, requires amino acids such as histidine, isoleucine, leucine, methionine, glutamate, glycine, tryptophan, valine (Murray et al., 1993) to achieve optimal

293 cultivation conditions. The results obtained with *E. faecalis* in the present work
294 confirm that the different amino acid and peptide composition of marine
295 peptones could procure different efficiency in the amino acid and peptide uptake
296 systems (Kunji, Mierau, Hagting, Poolman, & Konings, 1996). The increase of
297 nitrogen provided by SPHs, significantly arises glucose uptake, and
298 consequently lactic acid production (Table 4), and hence its assimilation, as has
299 already been reported by Fakas, Papanikolaou,Komaitis, and Aggelis (2008) for
300 *Cunninghamella echinulate*.

301 **3.3. *Effect of SPHs on bacteriocin like inhibitory substances production***

302 In order to investigate antagonistic profiles of the *E.faecalis* DM19 strain, cell-
303 free supernatant extracts were tested against 21 microbial strains (Table 5).
304 Differences in the diameter of inhibition halos were particularly notable up to
305 18.73±1.80 mm. Cell-free supernatants from *E. faecalis* DM19 grown in MRS
306 exhibited bactericidal activity against the majority of studied strains excluding
307 *Aspergillus niger* CECT 2088*, Bacillus coagulans* CECT 56*, Penicillium*
308 *expansum* DSMZ 62841 and *Yersinia enterocolitica* CECT 4315. However,
309 these microorganisms were sensitive to the cell-free supernatants from MRS
310 prepared by the hydrolysates (Table 5). Previous works had already reported
311 that *E. faecalis* produce a broad-spectrum of bacteriocins (enterocins), which
312 are active against Gram-positive and Gram-negative bacteria (Line, Svetoch,
313 Eruslanov, Perelygin et al., 2008; Pieniz, Andreazza, Anghinoni, Camargo, &
314 Brandelli, 2014). This fact could be reasonable since the pH of the culture
315 supernatants used in this study were neutralized to avoid the antimicrobial
316 effect of organic acids (lactate, acetate, etc.) and since anaerobic conditions
317 were imposed to decrease H_2O_2.

318 The substitution of commercial peptone in the media by one-fold concentration
319 of SPH did not lead to a relevant production of antimicrobial compounds in
320 terms of effectiveness and number of microorganisms inhibited. However, when
321 commercial peptone was substituted by two-fold concentration of SPHs, the
322 supernatants exhibited an interesting inhibitory activity against a large number
323 of indicator strains investigated (Table 5). The highest inhibitory activity was
324 observed mainly against *Listeria monocytogenes* CECT 4032, which was
325 significantly inhibited by SQES supernatant as compared with MRS ($p \leq 0.05$).

326 It has been shown that the composition of the growth medium, particularly the
327 source and concentration of nitrogen, strongly affects the production of
328 bacteriocin (Gänzle, Weber, & Hammes, 1999). As can be seen in Table 5, the
329 production of bacteriocin-like substances by *E. faecalis* production was
330 enhanced when SPHs concentration increased from 10 to 20 g/L. Accordingly,
331 the increasing concentrations of yeast extract, meat extract or peptone can
332 allow an increase in the production of bacteriocins. It can be possible that fish
333 peptones could have suitable contents, in terms of free amino acids and short
334 peptides, that are precursors for bacteriocin production (Kanmani et al., 2010),
335 whereas some authors have pointed out that bacteriocin production by lactic
336 acid bacteria is a growth-associated process (De Vuyst, Callewaert, & Crabbé,
337 1996).

338 **3.4. Bacteriocin genes identified in the *E. faecalis* DM19 genome**

339 Once the genome of *E. faecalis* DM19 was assembled, the presence of
340 bacteriocin genes was tested using the BAGEL3 algorithm. Four sequences
341 related with bacteriocin-like genes were identified but only two showed proper

342 length and amino acid identity higher than 99%: the enterolysin A and a
343 lantibiotic gene (Table 6). The annotation of the bacteriocins identified was
344 based on amino acid sequences homology (Supplementary data).

345 Enterolysin A is a heat-labile bacteriocin, firstly reported in *E. faecalis* LMG2333
346 and widely distributed among enterococci strains (Nilsen et al. 2003; Hickey et
347 al. 2003; Nigutova et al. 2007; Almeida et al. 2011). It belongs to the class III of
348 the bacteriocins and has a bacteriolytic mode of action over cell wall (Nilsen et
349 al, 2003). Our results are in line with the data already published about
350 enterolysin A in which it was described that inhibits growth of enterococci,
351 pediococci, lactococci and lactobacilli (Nilsen et al, 2003; Khan et al, 2013).
352 Furthermore, enterolysin A shows a wide spectrum of activity possibly due to
353 the cleavage of the peptide bond in the stem peptide and the interpeptide bridge
354 of the peptidoglycan units (Khan et al, 2013).

355 Besides, the other bacteriocin identified in *E. faecalis* DM19 is the one known
356 as lantibiotic because of the content of lanthionine and/or methyllanthionine in
357 its primary structure. These substances belong to class I bacteriocins and its
358 activity falls mainly on Gram-positive bacteria (Bierbaum et al, 2009).
359 Lantibiotics are heat-stable peptides and, like enterolysin A, also act on the cell
360 wall of other bacteria (Sahl et al, 1998).

361 **Conclusion**

362 Protein hydrolysates prepared mainly from squid and shrimp have sufficient
363 nutritional value to support growth and metabolite production of *E. faecalis*
364 DM19. The increase of nitrogen provided by these hydrolysates in culture media
365 increases glucose uptake, and consequently lactic acid production by this

366 strain. Moreover, the increase of fish peptones enhanced the bacteriocin
367 producing of *E. faecalis* DM19, which was especially active against *Listeria*
368 *monocytogenes*. These strain properties, together with the low price of protein
369 hydrolysates, make them suitable for use in the fermented food industry.

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