Mutagenicity and genotoxicity assessment of a new biopreservative product rich in Enterocin AS-48

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ABSTRACT

A biopreservative derived from the fermentation of a dairy byproduct by *Enterococcus faecalis* UGRA10 strainis being developed. This product possesses a strong and wide antibacterial spectrum mainly due to the presence of Enterocin AS-48 in its composition. To assess its potential as food additive, the mutagenicicity and genotoxicity has been assayed by means of the bacterial reverse-mutation assay in *Salmonella typhimurium* TA97A, TA98, TA100, TA102, TA1535 strains (Ames test, OECD 471) and the micronucleus test (MN) (OECD 487) in L5178Y/ Tk+/- cells. The results in the Ames test after exposure to the byproduct (6.75-100 µg/plate) with absence and presence of the metabolic activation system from rat liver (S9 fraction), revealed not mutagenicity at the conditions tested. For the MN test, the exposition to five enterocin AS-48 concentrations (0.2-1 µg/µL) was tested in the absence and presence of S9 fraction, with no evidence of genotoxicity. Negative results in the mutagenicity and genotoxicity assays point out the good safety profile of the byproduct and support its use as additive. Further toxicological studies are required before its approval and commercial application.

Keywords: Dairy extract, Enterocin AS-48, mutagenicity, genotoxicity, in vitro, food additive

1. INTRODUCTION

Several lactic acid bacteria (LAB) can produce bacteriocins, molecules that can be defined as antimicrobial peptides or proteins ribosomally synthesized produced by bacteria with potential to inhibit the growth of food-borne pathogenic and spoilage bacteria (Franz et al., 2007). The interest as natural biopreservatives of bacteriocins, and their potential applications, alone or in combination with other natural antimicrobials is increasing (reviewed in Des Field et al., 2018). The genus *Enterococcus* belongs to LAB and it can be found in many foods such as milk, cheese, fermented sausages or olives (Foulquié- Moreno et al., 2006). Moreover, a selection of *Enterococcu*s strains with promising health-promoting capacities has been used as probiotics in animal and human health promotion to develop the intestinal microbial balance (European Commission, 2011). Thus, some strains are currently used as therapeutic treatments marketed as Cylactins (Hoffmann-La Roche, Basel, Switzerland), Fargo 688s (Quest International, Naarden, The Netherlands), ECOFLOR (Walthers Health Care, DenHaag, The Netherlands), or Symbioflor 1 (SymbioPharm, Herborn, Germany). In addition, the production of potent bacteriocins (enterocins) is a trait frequently found in enterococcal strains which reinforce the probiotic potential of this group of lactic bacteria (Foulquié-Moreno et al., 2006).

Enterococcus faecalis UGRA10 strain has been isolated from a raw sheep's milk farmhouse cheese and can produce a fermentation product rich in Enterocin AS-48 (Cebrian et al., 2012). AS-48 is a circular bacteriocin which shows activity mainly against many Gram-positive bacteria highlighting *Listeria, Bacillus, Enterococcus, Planococcus, Mycobacterium, Corynebacterium and Nocardia*. Although it also affects various Gram-negative species, these are much less sensitive due to the outer membrane protective effect (Gálvez et al., 1989). Furthermore, the activity of Enterocin AS-48 against

flagellate protozoa (*Leishmania* and *Trypanosoma*) has been recently described (Abengózar et al., 2017; Martinez-Garcia et al., 2018). All these antimicrobial properties confer to the Enterocin AS-48 several biotechnological properties with application in the food industry. Among them, the most extensively investigated application is its use as food biopreservative, because currently, natural products such bacteriocins produced by LAB has been demanded by consumers instead of chemical additives with potential health risks (Baños et al., 2019a). This bacteriocin has a great potential to control bacterial growth in animal food such as meats, dairy products, seafood, and also vegetable-based food (Ananou et al., 2010; Baños et al., 2016; Baños et al., 2019b).

There are several studies describing the mechanism of action of AS-48, its molecular structure and efficacy as an antimicrobial (Sanchez-Barrera et al., 2003). However, there are very few studies carried out in relation to its toxicological profile so far. Specifically, Cebrian et al. (2019) have described a safe profile of this substance after conducting different *in vitro* and *in vivo* studies. These authors demonstrated that AS-48 exhibit low haemolytic activity in blood, it did not induce nitrite accumulation in non-simulated RAW macrophages and possess low cytotoxicity in several human cell lines.. *In vivo*, the assessment of AS-48 toxicity in zebrafish eggs was also performed and showed no visible anomalies after 24 and 48 h in embryos exposed to low doses (0.6–3.0 mM), and the lethal dose 50 (LD_{50}) was established between 3.0 at 6.4 µM. The authors also reported the absence of lymphocyte proliferation after skin sensitization in mice, and lack of toxicity in this experimental model (Cebrian et al., 2019). Also, the subchronic studies on AS-48 toxicity carried out feeding mice with enterocin for 90 days revealed the absence of any adverse effect on animal health (Baños et al, 2019a). However, there are no genotoxicity and mutagenicity studies, of key importance to evidence its safety prior its approval and commercialization as food additive.

Accordingly, the purpose of this research was to assess the mutagenic and genotoxic potential of a UGRA10 fermented product rich in Enterocin AS-48 through two different *in vitro* tests recommended by EFSA Scientific Committee: The bacterial reverse-mutation assay in five strains of *Salmonella typhimurium* (Ames test, OECD 471) which detects gene mutations in the absence and presence of the microsomal fraction S9; and the Micronucleus test (MN, OECD 487) on L5178Y Tk+/- cells that detects clastogenic and aneugenic chromosome aberrations in the absence and presence of the external metabolic activation system from rat livers, the microsomal fraction S9.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Lactic product fermented by the UGRA10 strain containing 10 µg/µL of Enterocin AS-48 was provided by DMC Research Center (Granada, Spain). Chemicals for different assays were supplied by Gibco (Biomol, Sevilla, Spain), Sigma -Aldrich (Madrid, Spain), C-Viral S.L. (Sevilla, Spain) and Moltox (Trinova, Biochem, Germany).

2.2. Bacteria Reverse Mutation Test (Ames test)

The Ames test was performed following the OECD Guideline 471 (2020) and Díez-Quijada et al. (2019). Five *Salmonella typhimurium* histidine-auxotrophic strains (TA97, TA98, TA100, TA102 and TA1535) obtained from TRINOVA BIOCHEM GmbH (Germany) were cultured following the supplier instructions. The mutagenic activity of the lactic fermented product was assessed in the absence and presence of the external metabolic activation system from rat livers (S9 fraction). According to OECD 471 guideline, each experiment was performed with five decreasing concentrations of Enterocin AS-48 (100-6.75 µg/plate) starting from its maximum concentration present in the lactic fermented product which did not present bactericidal properties. Also, a

negative control (distilled sterile water), solvent control (DMSO) and a positive control for each strain in accordance with the presence or absence of S9 fraction were included: 9-Aminoacridine (50 µg/plate) was the positive control for TA97A without S9 fraction; 2-Nitrofluorene (2-NF) (0.1 µg/plate) for TA98; sodium azide (NaN3) (1 µg/plate) for TA100 and TA1535; and mitomycin C (MMC) (2.5 µg/plate) for TA102. The positive control in the presence of S9 fraction was 2-aminofluorene (2-AF) (20 µg/plate) for all strains. At least 3 independent experiments were performed using triplicate plates for each test concentration. Results are expressed as revertant colonies and mutagenic indexes (MI).

2.3. Micronucleus test

This assay was performed according to the OECD guideline 487 (2016). L5178Y/ Tk+/- cells were seeded at a concentration of 2.0×10^5 cell/mL and treated with five different concentrations of Enterocin AS-48 (0.2-1 µg/µL) selected according to the OECD 487 guideline and checking previously the absence of cytotoxicity. Moreover, these concentrations are in accordance with the highest concentration of Enterocin AS-48 available in the lactic fermented product (1 µg/µl) and the maximun concentration of use in the food industry. The experiment was performed in the absence of S9 during 24 h and presence of S9 during 4 h. Moreover, negative control: RPMI medium, and positive controls: mitomycin C (0.0625µg/mL) and colchicine (0.0125µg/mL) for clastrogens and aneugenic damage respectively in the absence of S9 fraction, and cyclophosphamide (8µg/mL) in the presence of S9 fraction were employed. After these periods, cells were exposed to cythochalasin B (6 µg/mL) for 20 h to block cytokinesis and obtain binucleated cells. Then, cultures were centrifuged, and the sedimented cells were subjected to a hypotonic treatment with KCl. Afterwards, the cells were again centrifuged and fixed. The resultant pellets were resuspended, dropped on microscope slides, and

stained with Giemsa 10%. The frequency of binucleated cells with micronuclei (BNMN) and the nuclear division index (NDI) were analysed according to the recommendations of OECD 487 (2016) by analysing at least 2000 binucleated cells per concentration.

2.4. Statistical Analysis

The statistical analysis was performed with Graph-Pad InStat software (Graph-Pad Software Inc., La Jolla, CA, USA). The non-parametric Kruskal-Wallis test was employed to compare the exposed samples with the negative controls. Differences were considered significant at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, respectively.

3. RESULTS AND DISCUSSION

In general, the toxicological studies performed with bacteriocins are very scarce and are mainly focused on analysis of their haemolytic activity and cytotoxicity potential in tumour cell lines, such as the case of Enterotoxin AS-48 (Baños et al., 2019b; Cebrian et al., 2019).

Regarding the Enterocin AS-48, in the present work, no signals of mutagenicity during the Ames test performance have been observed. The increasing concentrations of Enterocin AS-48 did not induce changes in the number of colonies in any of the *S. typhimurium* strains with and without S9 fraction (Table 1). MI higher than 2 was not obtained in any of the assayed experimental conditions. Solvent control (DMSO) did not induce statistically significant changes versus the negative controls. All positive controls show very significant differences from negative controls $p < 0.01$. Regarding the MN test, the number of binucleated cells with micronucleus (BNMN) did not increase at any concentrations of Enterocin AS-48 assayed, and no significant differences were observed in comparison to the negative control with and without S9 fraction (Table 2). The NDI did not show any sign of toxicity without S9 fraction; however, with S9 fraction a

168 statistically significant increase of NDI was observed at the highest concentration (1
169 µg/µL) ($p < 0.05$). All positive controls show significant differences from negative
170 controls ($p < 0.001$).

171 The negative results obtained in these two tests evidence that Enterocin AS-48
172 lacks genotoxicity and mutagenicity. No previous studies have been found in the
173 scientific literature on this matter, as far as we know. Only an *in vitro* study reported the
174 antigenotoxic effects of LAB, prebiotics and products of their fermentation by application
175 of the comet assay in caco-2 cells (Nowak et al. 2015). The mechanisms involved in
176 genotoxicity reduction by pro- and prebiotics are still not fully understood.

177 Globally, and as it has been stated before, *in vitro* toxicity studies regarding
178 enterocins are scarce. Specifically, there are studies that show cytotoxic effects of nisin Z
179 in melanoma cells (Lewies et al., 2018), enterocin B, A + B in Hela, HT29 and AGS cells
180 (Ankaiah et al., 2018) or S37 in Caco-2/TC27 cells (Belguesmia et al., 2011). Nowadays,
181 the commercialized bacteriocin most widely used as food preservative is Nisin (E234), a
182 bacteriocin naturally synthesized by *Lactococcus lactis*. Regarding Enterocin AS-48,
183 Cebrian et al. (2019) had carried out *in vitro* studies to know its cytotoxicity through the
184 MTT assay in CCD18Co and MCF10A cells, using 0.19 mg / mL as the highest
185 concentration and it did not produce any decrease in cell viability. In the present work,
186 up to 500 times higher concentrations (100 mg/mL in the MN assay) have been used and
187 no signs of genotoxicity have been observed.

188 On the other hand, the *in vivo* testing of AS-48 is very limited. A study performed in mice
189 intraperitoneally administered (i.p.) with 5 mg/kg AS-48 (100 µg/mouse) in 6 doses (one
190 every 8 h) revealed that this enterotoxin did not produce toxic effects, such as changes in
191 body mass or splenomegaly (Cebrián et al., 2019). In addition, a subchronic study (90-
192 days) of enterocin AS-48 in rat, indicated the absence of clinical symptoms, and no

differences in biochemical and haematological parameters in rats exposed to 200 mg/kg Enterocin AS-48 (Baños et al., 2019a). EFSA recommends a gradual approach to the generation and evaluation of information on the genotoxic potential of food additives that begins with a core battery of *in vitro* tests including a bacterial reverse mutation assay and *in vitro* assay MN test (EFSA, 2012). Additionally, one *in vivo* study must always be provided even if all of the *in vitro* studies are negative (EFSA, 2017). Despite to the absence of genotoxicity and mutagenicity of this fermentation product based on Enterocin AS-48, further toxicological studies such as *in vivo* studies are needed in order to demonstrate its safety, before its commercial application.

5. CONCLUSION

The lactic *E. faecalis* UGRA 10 fermented product rich in Enterocin AS-48 has no mutagenic and genotoxic effects when *Ames* and Micronucleus tests have been applied, with and without S9 fraction. However, further toxicological studies must be carried out for its authorization in order to demonstrate its safety prior its commercial application.

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309 Table captions:

310 **Table 1.** Results of the Ames test obtained from the lactic UGRA10 fermentation product
311 (based on Enterocin AS-48) in three independent experiments by triplicate. Milli Q water
312 was used as negative control (100 µl) and DMSO (10 µl) as solvent for positive controls.
313 Data are given as mean ± SD revertants/plate. Positive controls without S9 for TA97A:
314 9-aminoacridine (50 µg/plate), TA98: 2-nitrofluorene (0.1 µg/plate), TA100 and
315 TA1535: NaN3 (1.5 µg/plate) and TA102: mytomicin C (2.5 µg/plate). Positive control
316 for all strains with S9: 2-aminofluorene (20 µg/plate). ** $p<0.01$ very significant
317 differences from controls.

318 **Table 2.** The frequency of micronucleus (MN) and nuclear division index (NDI) in
319 cultured mouse lymphoma cells L5178-Y Tk+/- treated with the lactic UGRA10
320 fermentation product (based on Enterocin AS-48). The genotoxicity assay was performed
321 in absence and presence of the metabolic fraction S9. The values are expressed as mean
322 ± SD. The significance levels observed are * $p<0.1$ and *** $p<0.001$ in comparison to
323 control group values (negative control).

324324

Table 1

Table 1. Results of the Ames test of the Lactic fermentation product (Enterocin AS-48) in three independent experiments by triplicate. Milli Q water was used as negative control (100 µl) and DMSO (10 µl) as solvent for positive controls. Data are given as mean ± SD revertants/plate. Positive controls without S9 for TA97A: 9-aminoacridine (50 µg/plate), TA98: 2-nitrofluorene (0.1 µg/plate), TA100 and TA1535: NaN_3 (1.5 µg/plate) and TA102: mytomicin C (2.5 µg/plate). Positive control for all strains with S9: 2-aminofluorene (20 µg/plate). ***p*<0.01 very significant differences from controls.

Concentration (µg/plate)		TA97A				TA98				TA100				TA102				TA1535			
		-S9	MI	+S9	MI	-S9	MI	+S9	MI	-S9	MI	+S9	MI	-S9	MI	+S9	MI	-S9	MI	+S9	MI
Enterocin AS-48	Negative controls	193±18	-	179±76	-	19±6	-	22±1	-	124±12	-	87±17	-	255±73	-	190±35	-	15±2	-	14±4	-
	100	195±12	1.0	161±46	0.9	24±3	1.3	27±2	1.2	105±13	0.8	98±13	1.1	212±20	0.8	109±38	0.6	15±1	1.0	10±5	0.7
	50	195±13	1.0	238±55	1.3	18±4	0.9	23±3	1.1	86±6	0.7	94±11	1.1	280±26	1.1	116±13	0.6	11±1	0.8	12±2	0.9
	25	212±17	1.1	129±13	0.7	23±7	1.2	22±2	1.0	82±4	0.7	89±12	1.0	219±6	0.9	104±25	0.5	13±2	0.9	13±4	0.9
	12.5	200±18	1.1	205±45	1.1	17±2	0.9	21±3	1.0	79±9	0.6	87±16	1.0	281±14	1.1	100±9	0.5	14±3	0.9	12±5	0.8
	6.25	200±26	1.0	225±18	1.3	19±4	1.0	30±9	1.3	90±12	0.7	76±6	0.9	281±35	1.1	130±9	0.7	18±3	1.2	8±1	0.5
	Positive controls	583±49**	3.0	707±254**	4.0	1000±100**	53.6	242±43**	11.0	584±14**	4.7	631±117**	7.2	767±42**	3.0	473±31**	2.5	651±60**	44.4	659±39**	2.2
DMSO		220±6	1.1	268±17	1.5	24±2	1.3	12±3	0.6	78±2	0.6	95±3	1.1	193±25	0.8	204±4	1.1	17±5	1.2	17±1	1.2

Table 2

Table 2. The frequency of MN and NDI in cultured mouse lymphoma cells L5178-Y treated with the fermented product (based on Enterocin AS-48). The genotoxicity assay was performed in absence and presence of the metabolic fraction S9. The values are expressed as mean ± SD. The significance levels observed are * *p<0.1* and *** *p<0.001* in comparison to control group values (negative control).

Test substance	Absence of S9				Presence of S9			
	Treatment time (h)	**Concentrations**	**BNMN (%) ± SD**	**NDI ± SD**	**Treatment time (h)**	**Concentrations**	**BNMN (%) ± SD**	**NDI ± SD**
Negative control	24	-	0.7±0.2	1.4±0.1	3-6	-	0.7±0.1	1.6±0.0
Positive control	24	Mitomycin C 0.0625 µg/mL	2.7±0.6***	1.3±0.1	3-6	Cyclophosfamide 8 µg/mL	2.4±0.9***	1.7±0.1
		Colchicine 0.01 µg/mL	2.5±0.4***	1.4±0.0				
Enterocin AS-48	24	0.2 µg/µl	0.9±0.2	1.3±0.1	3-6	0.2 µg/µl	0.6±0.2	1.7±0.1
		0.4 µg/µl	0.9±0.3	1.4±0.1		0.4 µg/µl	0.7±0.1	1.6±0.0
		0.6 µg/µl	1.0±0.3	1.4±0.1		0.6 µg/µl	0.6±0.2	1.7±0.1
		0.8 µg/µl	0.9±0.1	1.4±0.1		0.8 µg/µl	1.1±0.1	1.7±0.1
		1 µg/µl	1.3±0.4	1.5±0.0		1 µg/µl	1.3±0.3	1.8±0.1*