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Comparative Application of Different Strategies of Bacteriocins Produced by *Carnobacterium maltaromaticium* MMF-32 for Inhibition of *Listeria monocytogenes* ATCC 19114 in Cold-Smoked Haddock

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Abstract

Cold-smoked haddock fillets (undyed) treated with bacteriocin producing *C. maltaromaticum* MMF-32, *C. piscicola* A9b bac$^-$ (a non-producer of bacteriocin) and supernatant, supernatant concentrated with $(NH_4)_2SO_4$ and semi-purified bacteriocin of *C. maltaromaticum* MMF-32 were challenged with *L. monocytogenes* ATCC 19114 (up to 2.2 x 10^3 CFU g^{-1}). Following treatment, samples were kept at 4 °C for 10 days. *L. monocytogenes* ATCC 19114, total bacterial and carnobacterium counts were determined along with changes in total volatile base nitrogen (TVBN) and biogenic amine production as well as texture, color and odor. The anti-listerial effect of *C. maltaromaticum* MMF-32 and *C. piscicola* A9b bac$^-$ did not work on the cold smoked haddock, this is because there were few *Carnobacterium* cells in the samples that were inoculated with 4 x 10^6 CFU g^{-1}, than the control *i.e* untreated fish samples and sample having only *L. monocytogenes* ATCC 19114. *C. maltaromaticum* MMF-32 did not show any spoiling capacity from the odour. All treated samples produced cadavarine. There is marked spoilage odour observed in samples treated with MRS supernatant and ammonium sulphate precipitated supernatant. During the period of storage, TVB-N treated samples exceeded the limit of 35 mg N 100 g^{-1}. The use of bacteriocins for inactivation of listeria cells, semi-purified bacteriocin showed a statistically significant reduction in *L. monocytogenes* ATCC 19114 growth on day 7. Although the study on anti-listerial effects of *C. maltaromaticum* MMF-32 was not successful, this organism did have a positive effect on retention of firmness and sensory perception in cold smoked haddock.

Keywords: Anti-listerial activity; Biogenic amines; Biopreservation; *Carnobacterium*; Cold-smoked haddock; Sensory analysis.

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1. Introduction

The development of effective processing treatments to extend the shelflife of fresh fish products is important [1]. Moreover, the consumer demand for high quality and minimally processed seafood has recently drawn great attention to this issue [2;3]. *Listeria monocytogenes* is a Gram-positive, rod-shaped, food-borne pathogen that exists in a wide range of seafood and lightly preserved fish products [4;5;6]. The ability to grow at low temperatures obviously presents a major challenge to food safety with regard to *L. monocytogenes* as this pathogen persists in food processing environments and proliferates during chilled food storage [7;8].

The occurrence of *L. monocytogenes* in raw fish fillets and smoked-fish has been demonstrated in many recent studies. Thus, in smoked-fish, the incidence of contamination by *L. monocytogenes* was *ca*. 30%, with populations of <100 CFU g^{-1} [9]. Reference [10] detected *L. monocytogenes* in raw fillets of catfish (23.5% of samples), trout (5.7% of samples), tilapia (10.3% of samples), and salmon (10.6% of samples). Furthermore, *L. monocytogenes* contamination is one of the leading causes of recalls in industrially processed foods due to microbiological safety concerns [8]. As this organism is capable of growth at refrigeration temperatures, the zero-tolerance ruling issued by the U.S. Food and Drug Administration and in the UK for *L. monocytogenes* in ready-to-eat foods presents serious challenges to the food industry. Cold-smoked fish products are of major concern due to the lack of heat inactivation during processing, and consumption without any cooking step [8;11].

Many preservation methods have been explored with the aim of reducing the incidence of *L. monocytogenes* in smoked fish. It is realised that salting selects Gram-negative, halophilic anaerobic bacteria, and a water activity of 0.95 does not inhibit *L. monocytogenes* development [12]. Moreover, refrigeration and vacuum storage do not guarantee inhibition of *L. monocytogenes* growth [13].

The inoculation of food with microorganisms or their metabolites, which have been selected for their antibacterial properties, may be an effective way of extending shelf life and food safety through the inhibition of spoilage and pathogenic bacteria without altering the nutritional quality of the food product. This is defined as biopreservation [14;15]. Additionally, the use of bacteriocin-producing cultures has an advantage of overcoming the decomposition and binding of food particles when used as additives [16]. The preservative effect of LABs is often due to the ability to produce inhibitory compounds, including hydrogen peroxide, ethanol, organic acids (lactic and acetic acid), carbon dioxide, bacteriocins or antibiotic-like substances [17;18]. Moreover, during storage, they naturally dominate the microbiota of many foods. Some LABs detected from seafood have been shown to have strong inhibitory activity against spoilage and pathogenic microorganisms, including *Listeria*, *Clostridium*, *Staphylococcus* and *Bacillus* spp. [19;20;21]. As a food additive, *Lactococcus lactis* producing nisin has been granted the generally regarded as safe (GRAS) grade for use in some foods [22], notably fermented products, but not yet in cold-smoked fish products.

Carnobacterium spp. have been studied for their role as a component of the protective flora in cold smoked salmon (CSS), because of the ability to grow in foods with low carbohydrate content, e.g. fish products [23;24]. Fortunately, there is not any evidence that carnobacteria have any effect on the sensorial properties of cold-

smoked salmon [25] in contrast to other bacteriocin-producing LABs [26]. In particular, workers have demonstrated the bio-preservative power of bacteriocin-producing LABs against *L. monocytogenes* in cold-smoked salmon [27;28;29]. Another strategy for applying bacteriocins for biopreservation of food is the use of ammonium sulphate precipitated supernatant containing bacteriocin [30]. Moreover, [30] demonstrated that the *C. piscicola* A9b bac⁻ nonbacteriocin producing strain inhibited *L. monocytogenes* and that a significant nonbacteriocin-dependent inhibition was functioning. However, work has so far not been performed on the bio-preservation of cold-smoked haddock using nonbacteriocin producing strains, bacteriocin producing strains and bacteriocins. The aims of this study were to: evaluate the inhibitory effects of *C. maltaromaticum* MMF-32, a bacteriocin producing strain, and a nonbacteriocin-producing mutant of *C. piscicola* A9b bac⁻ on the growth of *L. monocytogenes* ATCC 19114 in cold-smoked haddock. To further determine the anti-listerial effect of *C. maltaromaticum* MMF-32 bacteriocins on cold-smoked haddock using: *C. maltaromaticum* MMF-32 supernatant, *C. maltaromaticum* MMF-32 ammonium sulphate precipitated supernatant and *C. maltaromaticum* MMF-32 semi purified ammonium sulphate precipitated supernatant.

2. Materials and methods

2.1. Bacterial strains and culture media

C. maltaromaticum MMF-32 was isolated in this study from a sample of cold-smoked salmon. *C. piscicola* A9b bac⁻ (which does not produce bacteriocin) was obtained from Professor Lone Gram. *L. monocytogenes* ATCC 19114 was obtained from the American Type Culture Collection. All strains were maintained as 20% glycerol stock at -70 °C in TSB supplemented with 1% (w/v) sodium chloride. *C. maltaromaticum* MMF-32 was grown aerobically at 30 °C in de Man Rogosa and Sharpe (MRS) broth. *C. piscicola* A9b bac⁻ and *L. monocytogenes* ATCC 19114 were grown in TNB at 30 °C.

2.2. Production of crude, concentrated ammonium sulphate precipitated bacteriocin and semi-purified bacteriocin of C. maltaromaticum MMF-32

The crude preparations of bacteriocins were the supernatant fractions obtained after centrifugation (10,000 x *g*, 10 min at 4 °C) of 48 h culture of *C. maltaromaticum* MMF-32 grown in MRS at 30 °C.

Concentrated preparations of bacteriocins were prepared from crude preparations by precipitation with solid $(NH_4)_2SO4$ to a final concentration of 516 g l^{-1} at 4 °C with stirring. The precipitate was collected by centrifugation at 20,000 x *g* for 30 min at 4 °C, redissolved in 50 mM potassium phosphate buffer (pH 5.8) or distilled water, and filter sterilized (pore size, 0.45 µm; type Minisart NML; Sartorius) before its spectrum of activity was determined. The crude and concentrated ammonium sulphate precipitated bacteriocins were stored at -70 °C until use.

Semi-purified bacteriocin of *C. maltaromaticum* MMF-32 was obtained from concentrated preparations of bacteriocins using hydrophobic interaction chromatography.

The concentrated ammonium sulphate precipitate was equilibrated to the composition of binding buffer (50 mM

sodium phosphate and 1.0 M ammonium sulphate) by adjusting to pH 7.0 and filtered through 0.45 µm filters. The column was equilibrated with binding buffer (to rehydrate the column) (50 mM sodium phosphate and 1.0 M ammonium sulphate, pH 7.0) and the elution buffer (50 mM sodium phosphate, pH 7.0) at a flow rate of 1 ml min^{-1}. Sixty five ml of concentrated ammonium sulphate precipitate was applied to hydrophobic interaction chromatography (HIC) column Hi Trap™ Octyl sepharose FF column (1 ml) (AKTA Prime, Pharmacia), then eluted with elution buffer (50 mM sodium phosphate, pH 7.0) at a flow rate of 1 ml min^{-1} [31].

The absorbance was monitored at 280 nm, and bacteriocin activity of each fraction was determined by microtitre broth bioassay. The protein contents of the crude, concentrated ammonium sulphate precipitated bacteriocin and semi-purified bacteriocin of *C. maltaromaticum* MMF-32 were estimated with the Pierce BCA Protein Assay Kit and bovin albumin as standard (Thermo Scientific), using the method of [32]. Specific activity was noted in arbitrary activity units (AU) per gram of protein.

2.3. Inhibition of L. monocytogenes in cold-smoked haddock

L. monocytogenes ATCC 19114 and *C. piscicola* A9b bac^- were subcultured three times in their respective culture media at 24 h intervals, whereas *C. maltaromaticum* MMF-32 was subcultured at 48 h intervals (the differences in subculture times is due to their different growth rates). Cells were harvested by centrifugation, washed three times with sterile PBS (0.01 M phosphate, pH 7.2) and finally resuspended in PBS to obtain cell concentrations of approximately 2.2 x 10^5 CFU ml^{-1} for *L. monocytogenes* ATCC 19114, 4.0 x 10^8 CFU ml^{-1} for *C. piscicola* A9b bac^-, and 4.4 x 10^8 CFU ml^{-1} for *C. maltaromaticum* MMF-32.

Cold-smoked haddock fillets (undyed) were purchased from a retail shop in Scotland, cut into thin slices of 10 g ± 1.0 g weight and treated first by inoculating with 100 µl of 4.0 x 10^6 CFU g^{-1} fish of *C. maltaromaticum* MMF-32 and *C. piscicola* A9b bac^-. Fish slices were kept in a laminar–flow biological safety cabinet for approximately 10 min in order to dry off excess liquid, after which 100 µl of *L. monocytogenes* ATCC 19114 suspensions containing 2.2 x 10^5 CFU ml^{-1} (confirmed by plate drop count) were inoculated onto each sample to give a final inoculum of 2.2 x 10^3 CFU g^{-1} of fish.

Following treatment, samples were individually packed in sterile petri dishes and kept at 4 °C for 10 days; this was the first application test to determine the inhibitory effect of *C. maltaromaticum* MMF-32 and *C. piscicola* A9b bac^-on *L. monocytogenes* ATCC 19114 in cold-smoked haddock fillet before using the bacteriocins. For organoleptic evaluation, samples were prepared as above, with the exception that *L. monocytogenes* ATCC 19114 was not added (treatments E1 to F1).

The treatments are summarized in Table 1. A1 corrresponds to the addition of *L. monocytogenes* ATCC 19114 alone in cold-smoked haddock; B1 corresponds to the co-culture of *L. monocytogenes* ATCC 19114 with *C. maltaromaticum* MMF-32 in cold-smoked haddock; C1 is the co-culture of *L. monocytogenes* ATCC 19114 with *C. piscicola* A9b bac^- in cold-smoked haddock; D1 is the control-uninoculated cold-smoked haddock; E1 corresponds to the cold-smoked haddock inoculated with *C. maltaromaticum* MMF-32 only and F1 corresponds to cold-smoked haddock inoculated with *C. piscicola* A9b bac^-.

Table 1: Experimental treatments of cold-smoked haddock with *Carnobacterium* spp.

Treatment code	Agent added		
	L. monocytogenes ATCC 19114	C. maltaromaticum MMF-32	C. piscicola A9b bac$^-$
A1	+	-	-
B1	+	+	-
C1	+	-	+
D1 (control)	-	-	-
E1	-	+	-
F1	-	-	+

L. monocytogenes ATCC 19114 was added at 2.2 x 10^3 CFU g^{-1}

C. maltaromaticum MMF-32 culture was added at 4.0 x 10^6 CFU g^{-1}

C. piscicola A9b bac$^-$ was added at 4.0 x 10^6 CFU g^{-1}

Control sample for Table 1. is D1

Following the first application test, each haddock slice surface was inoculated with 36 µg of semi-purified peptide solution, (2.7 x 10^5 AU g l^{-1} protein) per g of fish or 0.5 ml of concentrated ammonium sulphate precipitated supernatant and (1.7 x 10^4 AU g l^{-1}) or MRS (6.72 x 10^3 AU g l^{-1}) culture supernatant.

Fish slices were kept in a laminar–flow biological safety cabinet for approximately 10 min in order to dry off excess liquid, after which 100 µl of *L. monocytogenes* ATCC 19114 suspensions containing 4.0 x 10^5 CFU ml^{-1} were inoculated onto each sample to give a final inoculum of 4.0 x 10^3 CFU g^{-1} of fish. For organoleptic evaluation, samples were prepared as above, with the exception that *L. monocytogenes* ATCC 19114 was not added (treatments F to H). Following treatment, the samples were individually packed in sterile petri dishes and kept at 4 °C for 10 days. The treatments applied are summarized in Table 2. A corresponds to the addition of *L. monocytogenes* ATCC 19114 alone in cold-smoked haddock; B corresponds to the co-culture of *L. monocytogenes* ATCC 19114 with MRS supernatant in cold-smoked haddock; C is the co-culture of *L. monocytogenes* ATCC 19114 with supernatant concentrated with $(NH_4)_2SO_4$ in cold-smoked haddock; D is the co-culture of *L. monocytogenes* ATCC 19114 with semi-purified bacteriocin in cold-smoked haddock; E corresponds to the control-uninoculated cold-smoked haddock; F corresponds to the cold-smoked haddock inoculated with MRS supernatant only; G is the cold-smoked haddock inoculated with supernatant concentrated with $(NH_4)_2SO_4$ only, and H is the cold-smoked haddock inoculated with semi-purified bacteriocin only.

Table 2: Experimental treatments of cold-smoked haddock with bacteriocins

Treatment code	Agent added			
	L. monocytogenes ATCC 19114[a]	MRS supernatant[b]	Supernatant conc. with $(NH_4)_2SO_4$[c]	Semi-purified[d]
A	+	-	-	-
B	+	+	-	-
C	+	-	+	-
D	+	-	-	+
E (control)	-	-	-	-
F	-	+	-	-
G	-	-	+	-
H	-	-	-	+

[a] *L. monocytogenes* ATCC 19114 was added at 4.0 x 10^3 CFU g^{-1}.

[b] Supernatant of *C. maltaromaticum* MMF-32 culture in MRS broth added at 6.2 x 10^3 AU g l^{-1}.

[c] Supernatant of *C. maltaromaticum* MMF-32 culture in MRS broth, concentrated with $(NH_4)_2SO_4$ (1.7 x 10^4 AU g l^{-1}).

[d] Semi-purified concentrated $(NH_4)_2SO_4$ supernatant added at 2.7 x 10^5 AU g l^{-1} of haddock (36 µg g l^{-1} of haddock).

Control sample for Table 2. is E

The experiments were performed twice, and samples were taken in duplicate at day 1 and periodically during the 10 days of storage for microbiological analyses and pH measurement, respectively. Texture, colour and organoleptic analyses were done at intervals of 3 days during the 10 days of storage (i.e. all the organoleptic studies for both experiments).

2.4. Microbiological analysis

Ten grams of each fish slice were aseptically placed into a sterile stomacher bag (Seward) and homogenised for 3 min in 90 ml volumes of *Listeria* primary selective enrichment broth base CM0863 supplemented with SR0142E (UVM I; Oxoid) using a Lab Blender 400 Stomacher (Seward). The homogenate was incubated at 30 °C for 24 h. One hundred µl volumes of (UVM I) homogenate were transferred to 10 ml volumes of *Listeria* secondary selective enrichment broth base CM0863 supplemented with SR0143E (UVM II; Oxoid) and incubated at 30 °C for 24 h. Serial dilutions were prepared to 10^{-8} in UVM II, and 0.1 ml amounts were spread

over the surface of triplicate plates of PALCAM agar base CM0877 supplemented with SRO150E (PALCAM; Oxoid) [for the recovery of *L. monocytogenes*] plates with incubation aerobically at 30 °C for 48 h. The total number of LABs were determined on All Purpose Tween agar (APT) (VWR) supplemented with $NaNO_2$ (0.6%), polymyxin B-sulphate (0.003 g l^{-1}) and actidione cycloheximide (0.01 g l^{-1}) all obtained from Sigma-Aldrich to form nitrite actidione polymyxin (NAP) agar [33]. Control samples were tested alongside others. Total viable bacterial counts were determined using TNA with incubation aerobically at 30 °C for 48 h. *Carnobacterium* spp. were selectively enumerated on cresol red thallous acetate sucrose agar (CTAS) as proposed by [34], with some modification. Thus, the inoculated plates were incubated at 25 °C for 48 h for LAB, and followed by 48 h at 15 °C instead of 48 h at 8°C, as proposed by [34].

2.5. Total volatile base nitrogen (TVBN)

The steam distillation method of [35] was used. Thus, 200 ml of 7.5% aqueous trichloroacetic acid solution was added to 100 g of fish muscle in a metal beaker and homogenized in a Waring blender before the mixture was filtered through Whatman No 3 filter paper. The extract was stored in a cooler for one week. Using a Kjeldahl-type distillator (Struer TVN), steam distillation was performed by transferring 25 ml of filtrate into a distillation flask followed by 6 ml of 10% NaOH. Ten millilitre of 4% boric acid (containing 0.04 ml of methyl red and bromocresol green indicator) was pipetted into an Erlenmeyer flask and placed under the condenser for the titration of ammonia. Distillation was started and continued until a final volume of 50 ml was obtained in the beaker (40 ml of distillate). The boric acid solution turned green when alkalinized by the distilled TVB-N, which was titrated with aqueous 0.025 N sulphuric acid solutions using a 0.05 ml graduated burette. Complete neutralization was obtained when the colour turned grey/pink on the addition of a further drop of sulphuric acid.

2.6. Biogenic amine measurement

Approximately 50 g of fish sample was weighed into a glass container and homogenized for 45 sec in *ca*. 100 ml 10% TCA. The extract was filtered through Whatman 542 filter paper under vacuum and made up to 100 ml in a volumetric flask, mixed thoroughly, and then a small amount was filtered through a 0.45 µm filter. Derivatization was determined by adding 0.25 ml of sample/standard to 0.5 ml of o-phthaldialdehyde (OPA) reagent in a test tube with a screwed cap. The solution was kept in dark for exactly 3.5 min. Then 2 ml of ethyl acetate was added and vortexed for 1 min and kept until phase separation was completed. A volume of 1.5 ml from the top phase was pipetted into a vial and kept for 3.5 min. After the addition of ethyl acetate, the sample/standard was injected for analysis. Approximately 100 mg of each standard was weighed and made up to 100 ml with 10% TCA in volumetric flasks. A stock solution was made by mixing the four amines (histamine, tyramine, putrescine and cadaverine) in volumes, which were about 10 mg of the amines 100 ml^{-1} of 10% TCA, the amount is for each of the amines. Suitable dilutions were then made for standard curve preparation. Quantification of samples was by area measurement determined from a standard area versus concentration plot [36,37;38;39;40;41;42;35].

2.7. Sensorial analysis

Postgraduate students from the Institute of Aquaculture at the University of Stirling, Scotland, were asked to evaluate the acceptability of samples from duplicate trials of treatment D1 to F1 and E to H to compare them to the control (treatment D1 and E) with respect to colour, odour and texture. Scales for colour, odour and texture features were presented to the panelists. The sample was considered discoloured if the colour was pale. Odour was classified as 10-8 for 'smoke aroma', 7.8-4 for absence of smoke and 3.9-below as spoilage. Texture was scored qualitatively as either 'firm' (normal texture), slightly firm and pasty.

The cold-smoked haddock used for the present analysis was undyed cold-smoked haddock - this was to enable proper observation of changes in the organoleptic parameters.

2.8. Statistical analysis

Statistical analysis involved use of MINI TAB. Significant differences among treatment means for each parameter measured over the 10 days of storage of smoked haddock were tested by analysis of variance using the general linear model and comparisons with controls using Dunnet's test. Pair-wise comparisons were analyzed using Tukey's test for statistical significant differences, with a P value of < 0.005 considered significant.

3. Results

3.1. Broth assay: inhibition of L. monocytogenes ATCC 19114 by C. maltaromaticum MMF-32 culture supernatant, ammonium sulphate precipitated culture supernatant and semi-purified bacteriocin

The broth assay tests showed the inhibitory effects of supernatants from cultures of *C. maltaromaticum* MMF-32, ammonium sulphate precipitated supernatant and semi-purified bacteriocin on *L. monocytogenes* (Fig.1.). The addition of *C. maltaromaticum* MMF-32 cell-free supernatant inhibited the growth of *L. monocytogenes* ATCC 19114 at 8 h, but thereafter increased growth of *L. monocytogenes* was obvserved (Fig.1. A). A significant growth inhibition in the growth of *L. monocytogenes* ATCC 19114 was observed by ammonium sulphate precipitated supernatants of MMF-32 over 40 h of incubation (Fig.1. B). Inhibition was observed against *L. monocytogenes* ATCC 19114 following treatment with semi-purified bacteriocin of *C. maltaromaticum* MMF-32, only at 16 h post incubation (Fig.1. C).

3.2. Inhibition in the growth of L. monocytogenes ATCC 19114 on cold-smoked haddock following incubation with C. maltaromaticum MMF-32 and C. piscicola A9b bac⁻ during storage at 4 °C for 10 days

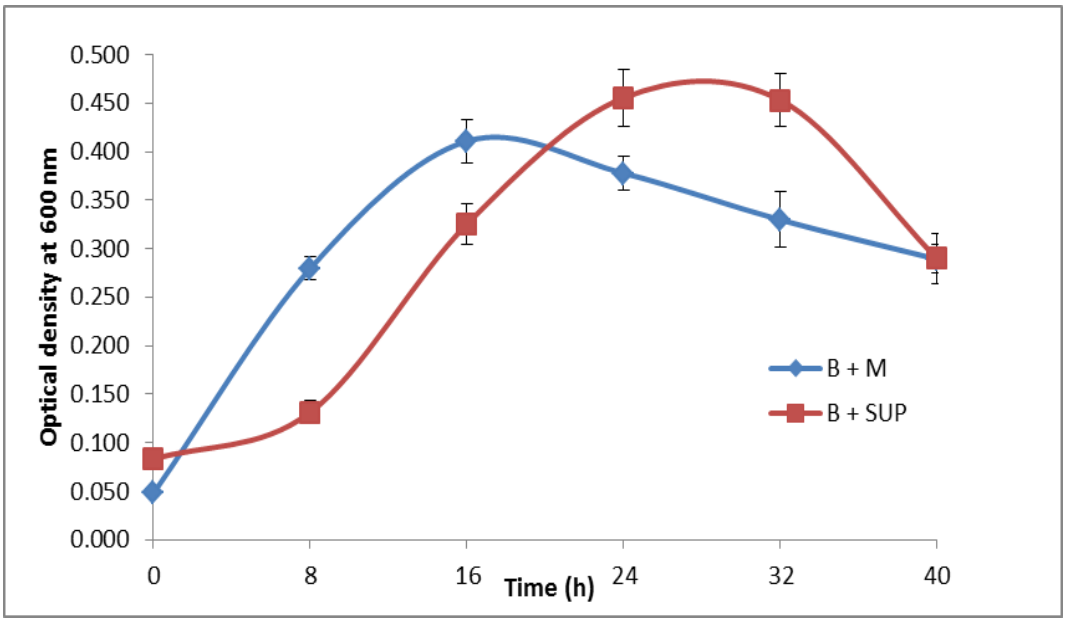
In this experiment there were 4 treatments (Table 1). These were cold smoked haddock with A1. *L.* monocytogenes ATCC 19114 added, B1. L. monocytogenes ATCC 19114 and C. maltaromaticum MMF-32 added, C1. L. monocytogenes ATCC 19114 and non-bacteriocin producing C. piscicola A9bac⁻ added and D1. Untreated control of cold smoked haddock did not have anything added to them. Initial counts of C. maltaromaticum MMF-32 and C. piscicola A9bac⁻ added were 4.0×10^6 CFU g^{-1}. However, at the first sampling time at 24 hours post inoculation, the count of Carnobacterium was higher in the treatments to which no *Carnobacterium* had been added (A1 and D1) than in the treatment to which it had been added. This

suggested that either there had been a problem with the inoculation or subsequent enumeration procedures, therefore the results from this experiment were discarded.

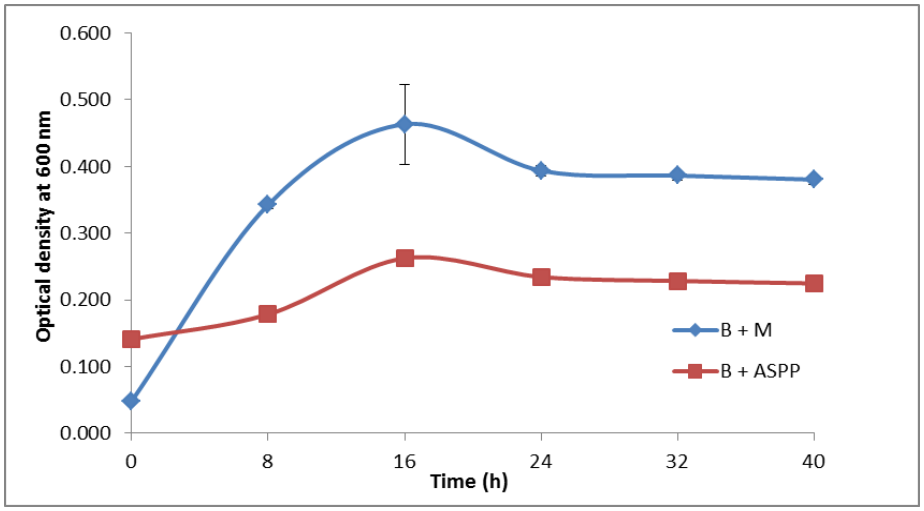
The *Carnobacterium* counts revealed that these were not significantly different, nor were there any significant differences between the treatment on day 3, 7 or 10. Although the control (D1) was significantly higher than the treatments on day 10 (Table 3).

Table 3: *Carnobacterium* counts on the days of inoculation

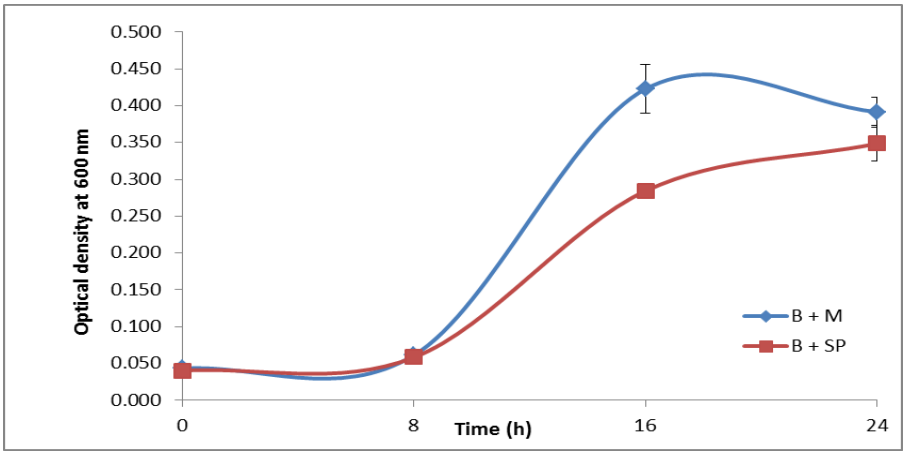
Days of inoculation	A1. *L. monocytogenes* ATCC 19114 added	B1. *L. monocytogenes* ATCC 19114 and *C. maltromaticum* MMF-32 added	C1. *L. monocytogenes* ATCC 19114 and non-bacteriocin producing *C. piscicola* A9bac⁻ added	D1. Untreated control of cold smoked haddock with nothing added.
1	1.75×10^9	8.38×10^8	4.63×10^8	2.13×10^9
3	5.75×10^9	1.25×10^{10}	1.38×10^{10}	1.25×10^8
7	2.37×10^8	2.25×10^8	2.63×10^8	1.25×10^7
10	1.23×10^8	1.93×10^8	2.00×10^8	7.68×10^8



A



B



C

Figures 1: Broth assay using 96-well microtitre plates showing inhibition of *L. monocytogenes* ATCC 19114 by (A) *C. maltaromaticum* MMF-32 culture supernatant (B) ammonium sulphate precipitated culture supernatant of *C. maltaromaticum* MMF-32 (C) semi-purified bacteriocin from ammonium sulphate precipitated culture supernatant of *C. maltaromaticum* MMF-32. B + M represents *L. monocytogenes* ATCC 19114 and media; B + SUP represents *L. monocytogenes* ATCC 19114 with media and supernatant bacteriocin; B + ASPP represents *L. monocytogenes* ATCC 19114 with media and ammonium sulphate precipitated supernatant; B + SP represents semi-purified bacteriocin. Points = Means ± SE.

3.3. Inhibition in the growth of L. monocytogenes ATCC 19114 on cold-smoked haddock following incubation with MRS crude supernatant, ASP supernatant and semi-purified bacteriocin during storage at 4 °C for 10 days

Fig. 2 shows the growth pattern of *L. monocytogenes* ATCC 19114 viable counts during storage of smoked haddock using different bacteriocin applications *i.e.* MRS crude supernatant (6.72 x 10^3 AU g l^{-1}), ASP

supernatant (0.5 ml; 1.7 x 10^4 AU g l^{-1}) and semi-purified bacteriocin (2.7 x 10^5 AU g l^{-1}; 36 µg l^{-1}) (Table 2.). *L. monocytogenes* ATCC 19114 alone (treatment A) grew rapidly in smoked haddock in the first 24 h, with counts increasing from 4.0 x 10^3 CFU g^{-1} initial inoculum (section 4.3.4.) to 5.3 x 10^8 on CFU g^{-1} day 1, and then similar levels were maintained until day 10 (4.7 x 10^8 CFU g^{-1}) during storage (Fig. 2). In the control samples (i.e. haddock only), *L. monocytogenes* ATCC 19114 was not observed, confirming that *L. monocytogenes* was introduced by the treatment (data not shown). A statistical significant reduction in *L. monocytogenes* ATCC 19114 counts was only observed by the application of semi-purified bacteriocin on day 7, resulting in a count of 8.59 log CFU g^{-1} (= 3.9 x 10^8 CFU g^{-1}) when compared with the positive control having count of 8.71 log CFU g^{-1} (=5.1 x 10^8) (treatment A). Neither the application of crude MRS supernatant nor ammonium sulphate precipitated supernatant (treatments B and C) resulted in inhibition in the growth of *L. monocytogenes* ATCC 19114 in the smoked haddock). The application of ammonium sulphate precipitated supernatant (treatment C) showed a statistical significant increase of 8.78 log CFU g^{-1} (= 6.3 x 10^8) of *L. monocytogenes* ATCC 19114 on day 7. The increase in count of *L. monocytogenes* ATCC 19114 in the ammonium sulphate precipitated supernatant may be due to variation in samples.

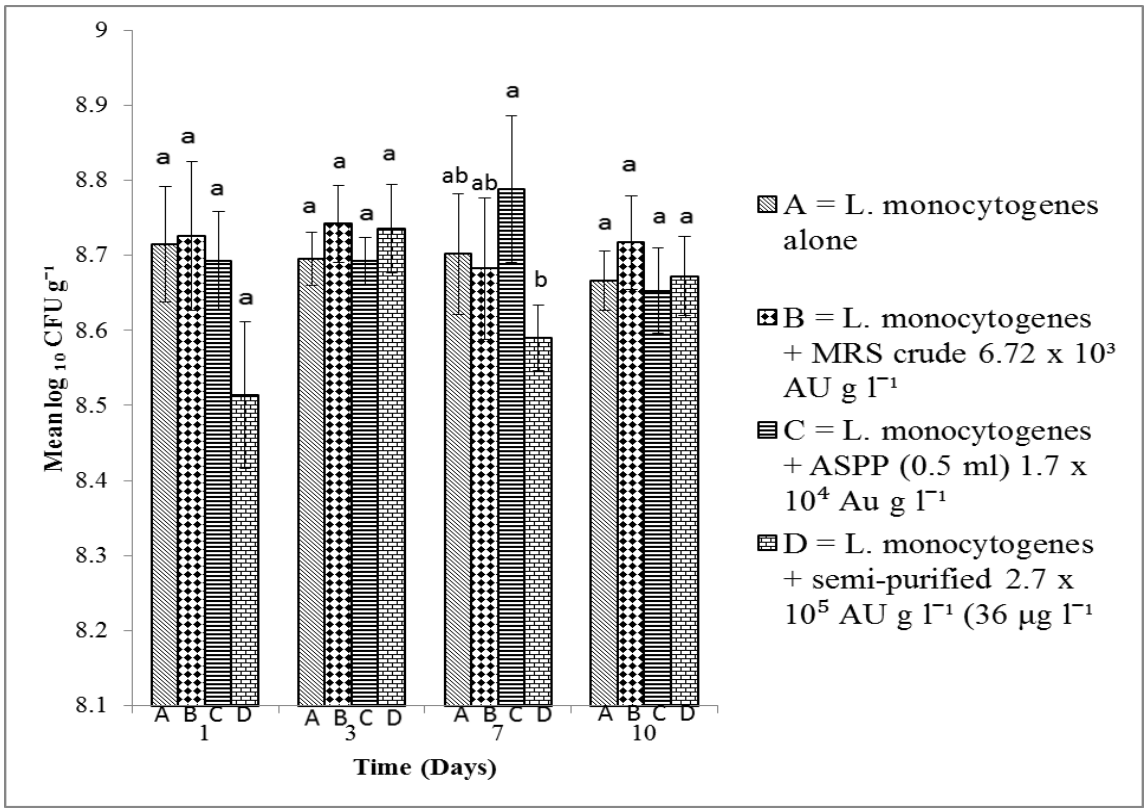


Figure 2: Total of viable counts of *L. monocytogenes* ATCC 19114 added to cold-smoked haddock stored at 4 °C for 10 days. (▨) *L. monocytogenes* (treatment A); (▩) *L. monocytogenes* with MRS supernatant (treatment B); (▤) *L. monocytogenes* with ammonium sulphate precipitated supernatant (treatment C) and (▥) *L. monocytogenes* with semi-purified bacteriocin (treatment D). The error bars indicate standard deviations of repeated treatments. Means ± STD with different low case within the same group of bars are significantly different ($P < 0.005$).

3.4. Total bacteria count on cold smoked haddock

This was carried out to determine the total bacteria count in the cold-smoked haddock and to determine any inhibition by the bacteriocins. Control samples gave counts from 2.0 x 10^7 to 1.9 x 10^8 log CFU g^{-1} during the storage peroid.

The total bacterial counts of the treated samples were high, ranging from 3.7 x 10^8 to 7.0 x 10^8 CFU g^{-1} over the 10 day storage period. Significant differences ($p \leq 0.05$) were observed between the treated samples (treatment A, B, C and D) and control samples (treatment E, untreated fish) during the storage period except for day 1 (Table 4).

However the appropriate controls (without *L. monocytogenes*) were not included and therefore the effect of the bacteriocins on total bacteria count could not be extrapolated.

Table 4: Total bacteria count on cold smoked haddock

Days of inoculation	A. *L. monocytogenes* ATCC 19114 alone	B. *L. monocytogenes* ATCC 19114 and MRS supernatant	C. *L. monocytogenes* ATCC 19114 and $(NH_4)_2SO_4$	D. *L. monocytogenes* ATCC 19114 and semi – purified	E. Control Untreated smoked haddock with nothing added
1	4. 7 x 10^8	5.6 x 10^8	4.5 x 10^8	5.8 x 10^8	1.9 x 10^8
3	5.7 x 10^8	7.0 x 10^8	5.5 x 10^8	5.1 x 10^8	1.3 x 10^8
7	4.9 x 10^8	5.6 x 10^8	4.9 x10^8	3.7 x 10^8	2.0 x 10^7
10	5.0 x 10^8	5.2 x 10^8	5.2 x 10^8	4.7 x 10^8	1.1 x 10^8

3.5. Biogenic amine production

MRS supernatant had the highest cadavarine content of 297.5 mg N 100 g^{-1}, at day 10 (treatment F) (Fig. 3). The uninoculated flesh had a lower cadavarine content of 83.5 mg N 100 g^{-1} (treatment E) whereas the cold-smoked haddock inoculated with ammonium sulphate precipitated supernatant and purified bacteriocin had ~60 mg N 100 g^{-1} (treatment G and H) of cadavarine on day 10. Putrescine content was detected only in MRS supernatant, having 61 mg N 100 g^{-1}, at day 10. Cadavarine and putrescine were not detected at day 0 from the bacteriocin inoculated and uninoculated flesh. Furthermore histamine was not detected from bacteriocin inoculated and uninoculated flesh on day 0 and 10 (data not shown). High levels of putrescine and cadavarine have been identified as potentiators of histamine or tyramine toxicity, but no recommendation about levels have been suggested [43].

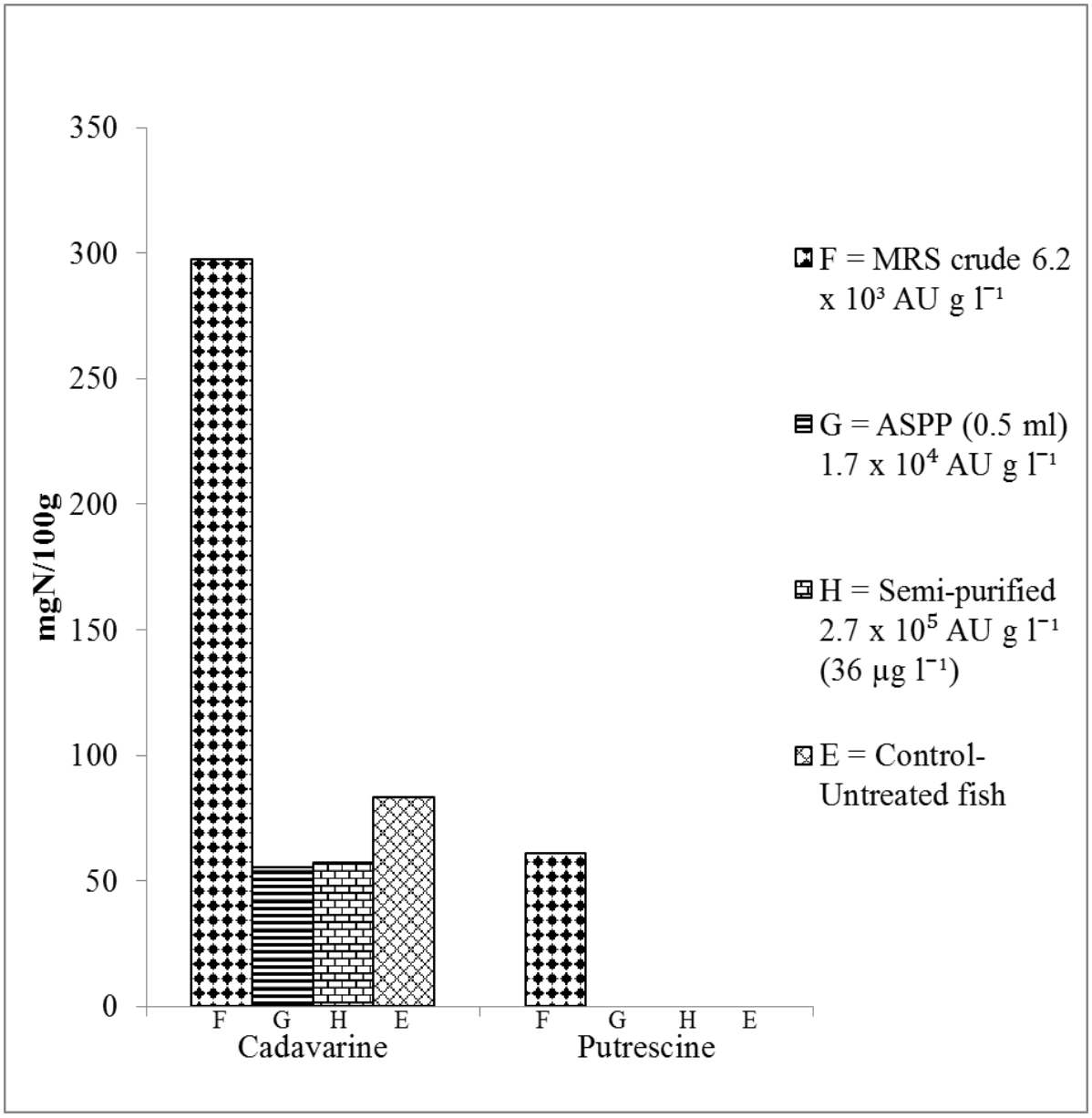


Figure 3: Biogenic amine concentration at day 10 in cold-smoked haddock during (storage at 4 °C). ( ) cold-smoked haddock with MRS supernatant (treatment F); ( ) cold-smoked haddock with ammonium sulphate precipitated supernatant (treatment G); ( ) cold-smoked haddock with semi-purified bacteriocin (treatment H) and ( ) control (treatment E, untreated fish).

3.6. Total volatile base nitrogen production

For all treatments excluding (treatment G), the volatile base nitrogen increased from 30 – 73 to 104 – 253 mg N 100 g^{-1} after 10 days of storage at 4 °C. Treatment G was exceptionally high values from 263-405 mg N 100 g^{-1} during the storage period. Significant differences were observed within treatments (Fig. 4.). The TVBN levels for all the treatments were above the acceptable limit for fresh fish appreciation.

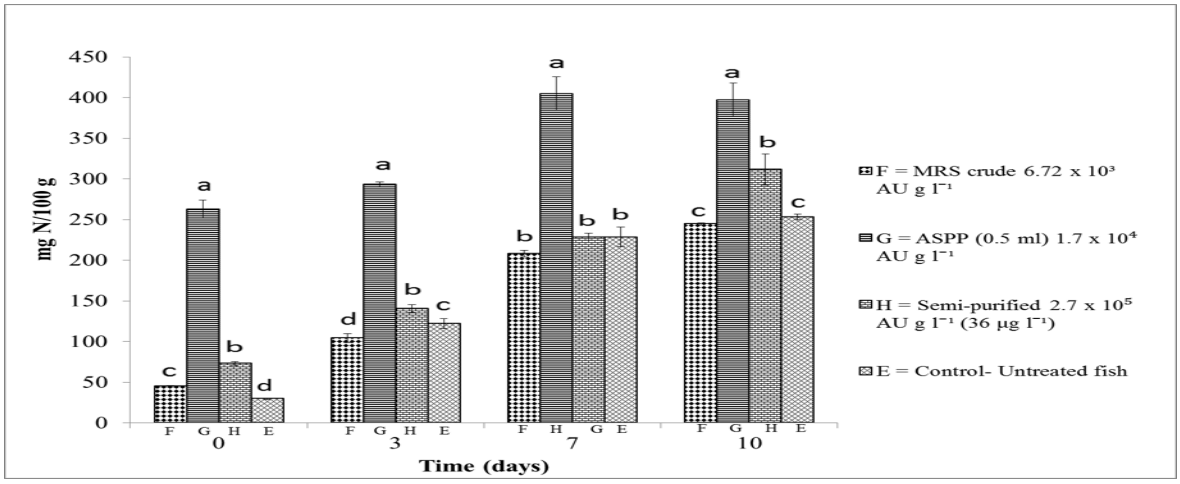


Figure 4: Total volatile base nitrogen production in cold-smoked haddock during storage at 4 °C for 10 days. ( ) cold-smoked haddock with MRS supernatant (treatment F); ( ) cold-smoked haddock with ammonium sulphate precipitated supernatant (treatment G); ( ) cold-smoked haddock with semi-purified bacteriocin (treatment H) and ( ) control (treatment E, untreated fish). The error bars indicate standard deviations of repeated treatments. Means that do not share a letter within the same group are significantly different ($P < 0.005$).

3.7. pH readings for Carnobacterium spp. and bacteriocin treated samples

There was no significant change revealed in the pH readings for the ten days of storage for any treatment conditions for *Carnobacterium* spp. and bacteriocin treated samples including the controls (Figs. 5. and 6.).

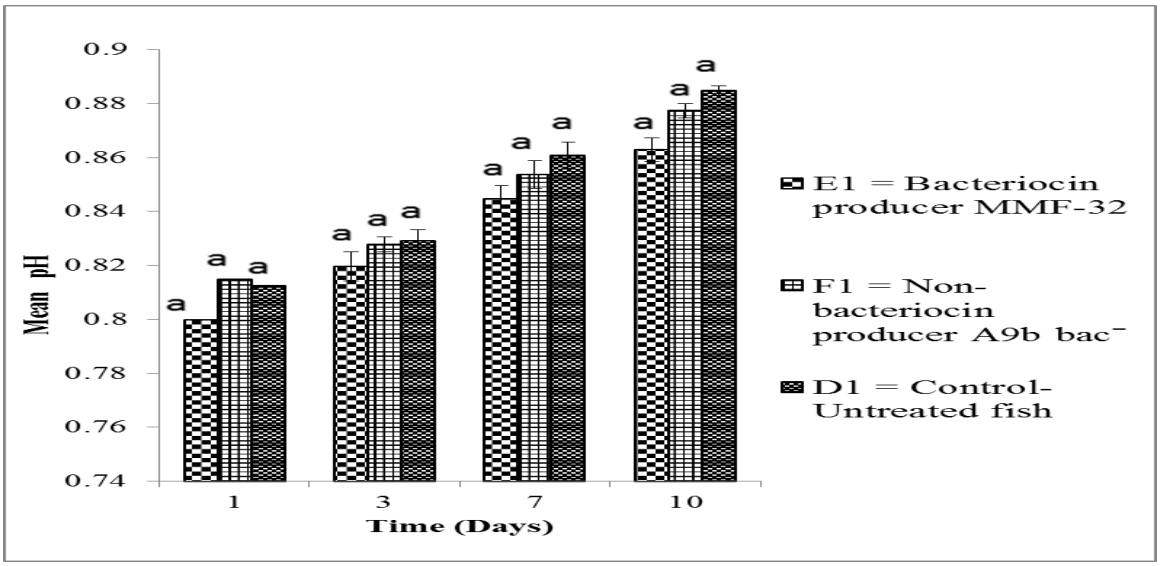


Figure 5: pH readings of *Carnobacterium* spp. treatment added to cold-smoked haddock stored at 4 °C for 10 days. ( ) *C. maltaromatic*um MMF-32 (treatment E1) and ( ) *C. piscicola* A9b bac⁻ (treatment F1); ( ) Control (treatment D1, untreated fish). The error bars indicates standard deviations of repeated treatments.

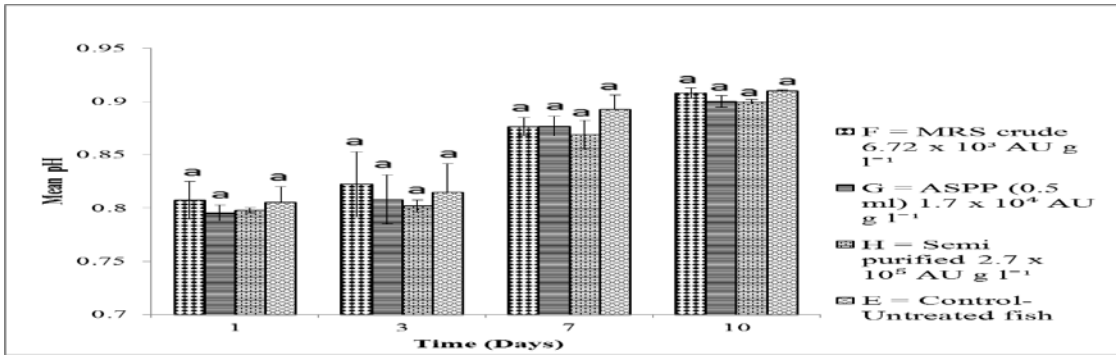


Figure 6: pH readings of all bacteriocin treatment added to cold-smoked haddock stored at 4 °C for 10 days. ( ) cold-smoked haddock with MRS supernatant (treatment F); ( ) cold-smoked haddock with ammonium sulphate precipitated supernatant (treatment G); ( ) cold-smoked haddock with semi-purified bacteriocin (treatment H) and ( ) control (treatment E, untreated fish). The error bars indicate standard deviations of repeated treatments.

The initial pH of smoked haddock was between 6.1 and 6.7 and did not get below 6.1 (Table 5. and 6.). On day 10 of storage, the pH readings of *Carnobacterium* spp. treated samples and bacteriocin applications varied between 7.22 to 7.78 and 7.84 to 8.19 respectively. Control is treatment D1 (untreated fish) for Table 5. Control treatment E (untreated fish) for Table 6.

Table 5: pH readings for *Carnobacterium* spp. treatment

Days	*C. maltaromaticum* MMF-32 Treatment E1	*C. piscicola* A9b bac⁻ Treatment F1	Control Treatment D1, untreated fish
1	6.37	6.56	6.44
	6.38	6.47	6.50
	6.23	6.55	6.58
	6.25	6.54	6.46
3	6.63	6.76	6.71
	6.38	6.72	6.66
	6.23	6.81	6.81
	6.25	6.62	6.81
7	7.09	7.08	7.24
	7.00	7.17	7.24
	6.94	7.14	7.30
	6.94	7.17	7.25
10	7.36	7.71	7.77
	7.22	7.69	7.78
	7.28	7.25	7.63
	7.31	7.52	7.51

Table 6: pH readings for bacteriocin treatment

Days	MRS supernatant Treatment F	Ammonium sulphate precipitated Treatment G	Semi-purified Peptide Treatment H	Control Treatment E untreated fish
1	6.19	6.14	6.27	6.19
	6.21	6.16	6.31	6.23
	6.58	6.34	6.24	6.53
	6.71	6.34	6.30	6.62
3	6.26	6.14	6.24	6.17
	6.24	6.14	6.29	6.21
	7.03	6.71	6.41	6.89
	7.09	6.75	6.41	6.88
7	7.62	7.42	7.59	7.94
	7.67	7.39	7.60	7.99
	7.35	7.74	7.15	7.47
	7.47	7.58	7.26	7.85
10	8.10	7.89	7.90	8.13
	8.19	7.84	7.92	8.15
	8.09	8.03	7.96	8.10
	7.98	8.04	7.98	8.14

3.8. Organoleptic parameters

C. maltaromaticum MMF-32 treated samples (treatment E1) were firm in texture throughout the storage period; *C. piscicola* A9b bac$^-$ treated samples (treatment F1) showed firmness from day 0 to 7; the control samples (treatment D1, untreated fish) were firm from day 0 to 3 (Table 7.). *Carnobacteriocin* spp. treated samples (treatment E1 and F1) retained the light pink colour during the first 7 days of storage, whereas the control sample (treatment D1) retained a light pink colour from day 0 to 3 (Table 7.). The *C. maltaromaticum* MMF-32 treated samples (treatment E1) retained the smoky odour for the first 7 days of storage, but an absence of the smoky odour was recorded on day 10. *C. piscicola* A9b bac$^-$ and control treated samples (treatment F1 and D1) had the smoky odour from day 0 to 3; on day 7 an absence of the smoky odour was noted (Table 7.).

Table 7: Organoleptic parameters of *Carnobacterium* spp. treated samples

Treatment	Days	Colour	Odour	Texture
C. maltaromaticum MMF- 32	0	Light pink	9.80	Firm
	3	Light pink	9.20	Firm
	7	Light pink	8.25	Firm
	10	Pale pink	7.20	Firm
C. piscicola A9b bac$^-$	0	Light pink	9.55	Firm
	3	Light pink	8.88	Firm
	7	Light pink	7.45	Firm
	10	Pale pink	3.95	Slightly firm
Control – untreated fish	0	Light pink	9.40	Firm
	3	Light pink	8.32	Firm
	7	Pale pink	4.40	Slightly firm
	10	Pale pink	3.6	Pasty

For odour:

10 - 9 represents smoky odour

8.9 - 8 represents smoky odour

7.8 - 4 represents absence of smoky odour

3.9 – and below represents spoilage

Texture was scored qualitatively as either 'firm' (normal texture), slightly firm and pasty.

The cold-smoked haddock used for the present analysis was undyed cold-smoked haddock, this is to enable proper observation of changes in the organoleptic parameters.

All the bacteriocin treated samples did not reveal any difference in firmness at day 0 (Table 8.). The MRS supernatant (treatment F) did not affect flesh firmness. The semi-purified bacteriocin (treatment H) retained firmness for 7 days. However, the ammonium sulphate precipitated supernatant (treatment G) and control (treatment E) decreased in firmness after day 3. The semi purified bacteriocin (treatment H) samples retained a light pink colour until day 7, unlike the control (treatment E, untreated fish) which was pale pink in colour on day 7. The MRS supernatant (treatment F), ammonium sulphate precipitated supernatant (treatment G) lost their light pink colour after day 3 (Table 8.). A change in the smoky odour was observed on day 10 in semi purified bacteriocin sample (treatment H). However, the samples treated with MRS supernatant (treatment F) had a spoilage odour from day 7 of storage; the ammonium sulphate precipitated supernatant and control samples (treatment G and E) had a spoilage odour on day 10 (Table 8.).

Table 8: Organoleptic parameters of bacteriocin treated samples

Treatment	Day	Colour	Odour	Texture	TVBN
MRS supernatant	0	Light pink	9.70	Firm	45 ± 0.8
	3	Light pink	7.50	Slightly firm	104 ± 5.4
	7	Pale pink	4.00	Pasty	208 ± 4.2
	10	Pale pink	3.00	Pasty	245 ± 1.3
Ammonium sulphate precipitated	0	Light pink	9.59	Firm	263 ± 11.3
	3	Light pink	8.14	Firm	293 ± 3.3
	7	Pale pink	6.50	Slightly firm	405 ± 20.7
	10	Pale pink	3.80	Pasty	397 ± 20.7
Semi-purified peptide	0	Light pink	9.50	Firm	73 ± 2.5
	3	Light pink	8.40	Firm	141± 4.5
	7	Light pink	7.58	Firm	229 ± 12.2
	10	Pale pink	6.86	Slightly firm	312 ± 19.0
Control–untreated fish	0	Light pink	9.40	Firm	30 ± 0.8
	3	Light pink	8.60	Firm	122 ± 6.0
	7	Pale pink	4.88	Slightly firm	229 ± 12.2
	10	Pale pink	3.56	Pasty	253 ± 3.5

For odour:

10 - 9 represents smoky odour

8.9 - 8 represents smoky odour

7.8 - 4 represents absence of smoky odour;

3.9 - below represents spoilage

For odour:

10 - 9 represents smoky odour

8.9 - 8 represents smoky odour

7.8 - 4 represents absence of smoky odour;

3.9 - below represents spoilage

Texture was scored qualitatively as either 'firm' (normal texture), slightly firm and pasty.

The cold-smoked haddock used for the present analysis was undyed cold-smoked haddock, this is to enable proper observation of changes in the organoleptic parameters.

A B

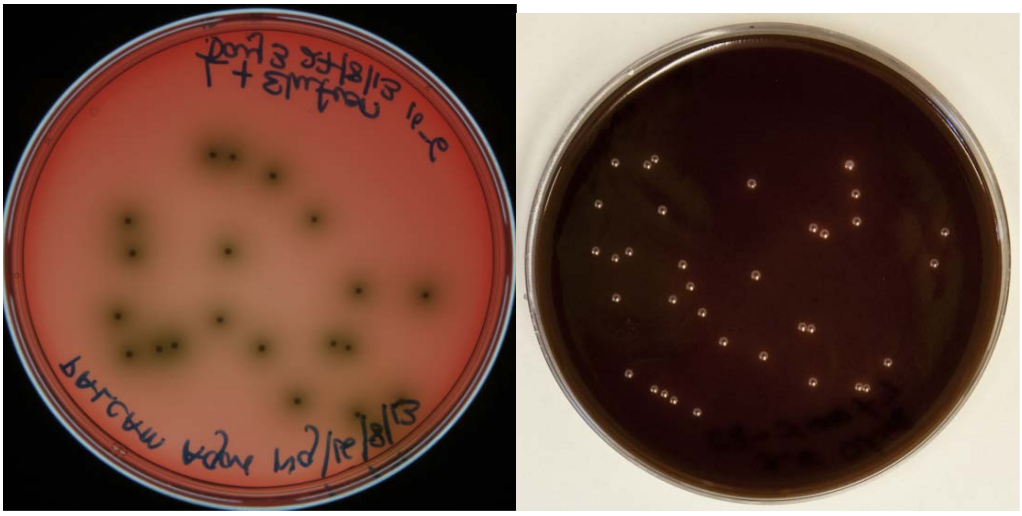


Figure 7: Recovery of *Listeria monocytogenes* ATCC19114 from cold-smoked haddock on polymyxin-acriflavin-lithium chloride-ceftazidime-aesculin-mannitol (PALCAM) agar (Panel A) and recovery of *Carnobacterium* spp. from cold-smoked haddock on cresol red thallium acetate sucrose inulin (CTSI) agar (Panel B).

4. Discussion

Carnobacteria are commonly found in chilled fresh and lightly preserved seafood. The presence of *C. divergens*

and *C. maltaromaticum* has been demonstrated for modified atmosphere-packed (MAP) coalfish, cod, pollack, rainbow trout, salmon, shrimp and surubim [44;45;46,47]). The ability of *Carnobacterium* spp. to grow

and produce bacteriocins with high anti-listerial activity at low and high sodium concentration has focused the attention of food scientists [48]). In this study, the broth assay demonstrated a reduction in the growth of *L. monocytogenes* ATCC 19114 (Figs. 1 A, B and C). The culture was sensitive to *C. maltaromaticum* strain MMF-32 supernatant, ammonium sulphate precipitated supernatant and semi-purified bacteriocin. *C. maltaromaticum* MMF-32 cell-free supernatant and ammonium sulphate precipitated supernatant inhibited the growth of *L. monocytogenes* ATCC 19114 until 8 h and 40 h incubation, respectively (Figs. 1 A and B). Addition of the semi-purified bacteriocicin only showed inhibition in *L. monocytogenes* ATCC 19114 growth at 16 h. Although OD and not CFU was used to quantify this result, this method was also used by [49]) to determine the inhibitory effect of bacteriocin preparations of *Lactobacillus curvatus* FS47, *Lb. curvatus* Beef3 and *Pediococcus acidilactici* against wild-type *L. monocytogenes*. [50] demonstrated inhibition of *L. monocytogenes* and or *Staphylococcus aureus* by all tested LAB strains cell-free supernatant using OD measurements. [20] reported the inhibition of *L. monocytogenes* by ammonium sulphate precipitated supernatant, also using OD measurements.

In order to determine if the inhibitory activity of the supernatant might arise from the production of hydrogen peroxide and lactic acid by LAB, catalase and 2NaOH were added to the supernatant extracts to exclude hydrogen peroxide and lactic acid [51].

Several studies have demonstrated the inhibitory activity of *Carnobacterium* spp. bacteriocins of class IIa against *L. monocytogenes* isolated from food [52;30;53]. In the current study, the activity of bacteriocin production in inhibiting *L. monocytogenes* ATCC 19114 was demonstrated by comparing the anti-listerial effects of *C. maltaromaticum* MMF-32 with nonbacteriocin producing *C. piscicola* A9b bac^- mutant of *C. piscicola* A9b bac^+ using cold smoked haddock stored at 4 °C for 10 days. The anti-listerial effect of *C. maltaromaticum* MMF-32 and *C. piscicola* A9b bac^- did not work on the cold smoked haddock, this is because there were few *Carnobacterium* cells in the samples that were inoculated with 4×10^6 CFU g^{-1}, than the control *i.e* untreated fish samples (treatment D1) and sample having only *L. monocytogenes* ATCC 19114 (treatment A1). This suggests there was a serious problem with some part of the protocol.

The observed non pH acidification in the present study confirms the non-acidic position of carnobacteria (Table 5). In the study, *C. maltaromaticum* MMF-32 cells were inoculated into the cold smoked haddock. According to [54] they demonstrated a non pH acidification of cold-smoked salmon blocks when inoculated with *C. divergens* V41, *C. piscicola* V1 or SF668. *C. maltaromaticum* MMF-32 did not show any spoiling capacity from the odour.

This agrees with the work of [23;55;25], that *Carnobacterium* spp. are not considered as spoilage organisms. *C. piscicola* A9b bac^- on cold-smoked haddock during the storage period showed a non pH acidification, spoilage odour was observed on day 10 along with the control samples (Tables 5. and 7.).

Firmness or hardness of flesh has been regarded as an important quality characteristic of fish product [56;57]. Texture is considered as one of the most significant parameters when the overall quality perception of fish product is being determined [58]. Reference [59] defined food texture as a collective term that covers several related physical properties. Reference [60] demonstrated that reduced moisture content, leads to increased texture firmness in smoked trout. Water content and lipid content of fish muscles determines the textural characteristics. The firmness observed during the period of storage by samples inoculated with *C. maltaromaticum* MMF-32 might be due to moisture loss thus resulting to a more tightly packed myofibrillar structure. Reference [29] demonstrated firmness in cold-smoked salmon treated with different strategies of divergicin M35 for three weeks. In the present study cold-smoked haddock samples treated with *C. piscicola* A9b bac$^-$ and control lost their firmness on days 10 and 7, respectively. Thus showing that the trend to reduce or remain constant depends on the application or treatment [29]. According to [61] these changes in texture may be due to different increased proteolytic activities by microbial enzymes specific *C. divergens* strains or endogenous spoilage flora.

The effect of cell-free supernatant, ammonium sulphate precipitated supernatant and semi-purified bacteriocin by the producing strain for inactivation of *L. monocytogenes* ATCC 19114 in cold-smoked haddock at 4 °C revealed; rapid growth of *L. monocytogenes* ATCC 19114 on smoked haddock when inoculated alone might be due to haddock being highly perishable, its scanty connective tissue content and loose meat tissue [62].

The inoculation of fish products or any type of food product with purified antimicrobial agent is liable to food preservative legislation. The addition of bacteriocins to cold-smoked haddock to the best of our knowledge has not been demonstrated. Semi-purified bacteriocin had a bacteriostatic effect on the growth of *L. monocytogenes* ATCC 19114 inoculated in smoked haddock, as seen on Fig. 2 on day 1, but this was not statistically significant. There was growth on day 3, but no statistically significant difference in growth of *L. monocytogenes* ATCC 19114 ($P > 0.005$) was observed in any of the treatments, until day 7. A significant difference was observed between treatment with the semi-purified bacteriocin and ammonium sulphate precipitated supernatant on day 7 (Fig. 2). Delay in growth was observed by [29] when using divergicin M35 from *Carnobacterium divergens* M35 to

inactivate *L. monocytogenes* added to cold-smoked salmon stored at 4 °C. [63] observed a delay also in growth when using sakacin P from *Lactobacillus sakei* on *L. monocytogenes* added to cold-smoked salmon stored at 10 °C. [64] demonstrated the same delay in growth when semi-purified bacteriocins from *Carnobacterium* spp. V41 was used as co-culture with *L. monocytogenes* added to cold smoked salmon stored at 8 °C. The use of MRS supernatant (treatment B) (Fig. 2) had no effect on growth reduction of *L. monocytogenes* ATCC 19114 added to cold smoked haddock during the storage period. In contrast, Reference [65] demonstrated a reduction of *L. innocua* level greater than 3 log cycles obtained on samples treated with 5% (v/v) supernatant V41 from *C. divergens* 41 added to cold smoked salmon trout during the storage at 5 °C after 1 week. The report of [29] revealed a rapid in activation of *L. monocytogenes* by divergicin M35 in culture supernatant and persistence in the salmon flesh longer (over 15 days). In the current study, the application of ammonium sulphate precipitated supernatant (treatment C) had a growth reduction of 0.1 log CFU g^{-1} of *L. monocytogenes* ATCC 19114 when added to cold smoked haddock during the storage period on day 1. A statistically significant difference was

observed on the growth of *L. monocytogenes* ATCC 19114 viable counts in co-culture with semi-purified bacteriocin on day 7 of the storage. Reference [30] demonstrated a decline of 3.5 log in viable count of listeria cells when precipitated carnobacteriocin (1024 BU ml^{-1}) from wild-type strain was co-cultured on cold-smoked salmon after day 6.

The effectiveness of using bacteriocins in food is limited by intrinsic and extrinsic factors related with a food product. These limiting factors are the inactivation of food components by proteases, lipids, microorganisms, packaging due to their molecular properties or their activity could be affected during processing (i.e. temperature and drying) [64;63;66]. Consequently, Reference [67] reported that the actual bacteriocin activity in the environment of the bacteria is much lower than expected. Resistance-development among target bacteria is among the factors that limit use of bacteriocins [68;69]. Various studies have revealed that the microbial flora of haddock comprised *Pseudomonas* spp., *Shewanella* spp., *Aeromonas* spp., *Vibrio* spp. and *Ph. phosphoreum* [70;71;72]. In cold smoked and vacuum packed fish products, Reference [73] reported that *Lactobacillus* species are present in the largest number and therefore are the most important microorganisms for sustained shelf life and as well as sensory characteristics. Reference [29] revealed that although they increased progressively in all samples during storage the cold-smoked salmon was still acceptable to the trained panelists until the third week of storage. The present study showed that by day 7 some of the treated samples and controls had been rejected by the sensory panelists (Table 8.), because of very strong amine off-odours. *Lb. curvatus* and *Lb. sakei* species often dominate the LAB colonizing lightly preserved fish products, although not specifically identified in this study [74;75,1998;76;77,78]). The total viable bacterial count analysis was carried out to determine the total aerobic bacterial count of the cold smoked haddock analysed. The cold-smoked haddock used in this study, revealed total aerobic flora from 2.0×10^7 to 1.0×10^8 CFU g^{-1} in control samples. The effect of bacteriocins on total bacterial counts could not be determined as the appropriate controls (without *L. monocytogenes*) were not included in this experiment. With regards to safety, all treated samples produced cadavarine, MRS supernatant treated samples only produced putrescine (treatments F) (Fig 3.) [77,78] reported that cadavarine and putrescine are often correlated with spoilage. They reported the production of biogenic amines primarily by *Ph. phosphoreum* in vacuum-packed cold-smoked salmon where agmatine (160-220 mg kg^{-1}), cadaverine (260-470 mg kg^{-1}), histamine (100-220 mg kg^{-1}) and tyramine (50-130 mg kg^{-1}) were formed at 5 °C. None of the treatments were able to produce histamine, which is known as the main agent for scrombroid fish poisoning [79]. Tyramine was not produced by any of the treated samples. Tyramine may cause migraine headaches and hypertensive effects, and in some cases can act as an existing possibility for measuring histamine [80]. Cadavarine was detected in the control sample (treatment E). According to [19,81] the most effective methods for stopping biogenic amines formation are handling and processing under sanitised and temperature control (<5 °C) conditions throughout the process. In this study there is marked spoilage odour observed in samples treated with MRS supernatant, ammonium sulphate m precipitated supernatant and controls (treatments F, G and E). Based on the fixed TVB-N limit (35 mg N 100 g^{-1}) as quoted in the EU regulations for gadoids [82], only the control sample was within the limit on the day zero (Fig. 4.). During the period of storage, all treated samples exceeded the limit of 35 mg N 100 g^{-1}. TVB-N is only useful to detect advanced spoilage because values only begin to increase at later stages of storage [83;84]. In contrast to the results of the present study, spoilage was observed from day 3 when increase in the TVB-N was detected.

Reference [71] suggested that high TVB-N levels in haddock fillets at sensory rejection are related to high *Ph. phosphoreum* counts that had reached TVC levels (>log 8 g^{-1}) for most sample groups. The present study showed the TVC levels of control samples to be from (2.0 x 10^7 to 1.0 x 10^8 CFU g^{-1}). TVB-N is mainly a composition of TMA and ammonia [70]. According to [70], haddock samples did not reveal any increase in TMA content, but TVB-N increased and was therefore considered to be due to the production of ammonia. Furthermore, the above worker reported that the *Vibrio/Photobacterium* group were found on the flesh of haddock on the last sampling day on LH medium after 4 days of incubation at 15 °C. Changes were observed in the pH of bacteriocin treated and control samples, but were not statistically significant (Fig. 6 and Table 6.). The increase of pH in treated and control samples during storage at 4 °C after day 3 may be directly related to the multiplication of psychrotrophic and mesophilic microorganisms, and connected with the autolytic reaction, which gives rise to the production of basic compounds that increase the pH. Later on the proteolytic action of spoilage bacteria also stimulated the same effect [85].

5. Conclusion

The presence of *L. monocytogenes* in cold-smoked fish cannot be completely controlled, but should be reduced to a minimum by Good Manufacturing Practice (GMP). This study has revealed that the inoculation of cold-smoked haddock with *C. maltaromaticum* MMF-32 resulted in no changes in either firmness or sensory perception of the product. During the use of bacteriocins for inactivation of listeria cells, semi-purified bacteriocin showed a statistically significant reduction in *L. monocytogenes* ATCC 19114 growth on day 7. Although the study on anti-listerial effects of *C. maltaromaticum* MMF-32 was not successful, this organism did have a positive effect on retention of firmness and sensory perception in cold smoked haddock.

6. Constraints/Limitations

The inhibition in the growth of *L. monocytogenes* ATCC 19114 on cold-smoked haddock following incubation with *C. maltaromaticum* MMF-32 and *C. piscicola* A9b bac^- during storage at 4 °C for 10 days. The results revealed that there had been problem with the inoculation or subsequent enumeration procedures. This is because at the first sampling time at 24 hours post inoculation, the count of *Carnobacterium* was higher in the treatments to which no *Carnobacterium* had been added (A1 and D1) than in the treatment to which it had been added. Therefore the results from this experiment were discarded. The test on total bacteria count on cold smoked haddock revealed that the appropriate controls (without *L. monocytogenes*) were not included and therefore the effect of the bacteriocins on total bacteria count could not be extrapolated.

7. Recommendations

Drop counts of *Carnobacterium* cells to be inoculated should be carried out to ensure the number of cells inoculated into the samples. For the total bacterial count, controls without *L. monocytogenes* should be included, i.e MRS supernatant, $(NH_4)_2SO_4$ supernatant and semi-purified bacteriocin without *L. monocytogenes*. This is to determine any inhibition by the bacteriocins.

Acknowledgements

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