***In* Seafood research from fish to dish Quality, safety and processing of wild and farmed seafood**
edited by: J.B. Luten, C. Jacobsen, K. Bekaert, A. Saebø and J. Oehlenschläger
2006 : Pages 403-410

http://www.wageningenacademic.com/

Selection of non-tyramine producing *Carnobacterium* strains for the biopreservation of cold-smoked salmon

BRILLET Anne[1], MATAMOROS Sébastien[1], BLANCHET-CHEVROLLIER Christine[1], LEROI Françoise[2], PREVOST Hervé[1] and PILET Marie-France[1]

[1] Unité de Recherche Qualité Microbiologique et Aromatique des Aliments, ENITIAA, rue de la Géraudière, BP 82225, 44322 Nantes Cedex 3, France.
[2]Laboratoire de Sciences et Techniques Alimentaires Marines, IFREMER, rue de l'Ile d'Yeu, BP 21105, 44331 Nantes Cedex 3, France.

*: Corresponding author : PILET Marie-France Tel : +33 2 51 78 55 23 Fax : +33 2 51 78 55 20, email address : pilet@enitiaa-nantes.fr

Abstract:

Biogenic amines in fish products are the result of the decarboxylation of free amino-acids by microorganisms. In cold smoked salmon, tyramine is mainly produced by lactic acid bacteria of the genus *Carnobacterium* that are also interesting bacteria for the control of the risk of Listeria monocytogenes growth. In this study, we have screened a collection of *Carnobacterium* strains that could be used for biopreservation in order to find a natural tyramine negative strain. This screening was performed using the detection by PCR test of a part of the tyrosine decarboxylase gene. On 35 strains of *Carnobacterium* tested, all showed the presence of the tdc gene suggesting that they all produce tyramine. This was assessed by the quantification of tyramine production for 10 strains. In a second part, a mutation procedure using ethyl methyl sulfonate was used to select a tyramine negative mutant of *Carnobacterium* divergens V41,that is a good candidate for biopreservation applications. A mutant strain called C. divergens V41A8 was selected and characterized. The mutant was identical to the wild strain concerning carbohydrates fermentation profile, antibiogram spectrum, bacteriocin production, and bacteriocin spectrum towards Listeria monocytogenes. The growth of strain C. divergens V41A8 was tested by comparison to the wild strain on a sterile cold smoked salmon model. The mutant grew more slowly than the wild strain on the product but it reached nearly the same level after 28 days of storage. Moreover, the production of tyramine detected on cold smoked salmon inoculated with C. divergens V41 (122 µg/g after 28 days of storage) was not detected at all when the product was inoculated with the mutant strain C. divergens V41A8. This strain could be an interesting alternative for the application of biopreservative *Carnobacterium* on food products naturally contaminated with tyramine such as smoked fishes.

1. Introduction

Biogenic amines are produced on several food products by decarboxylation of amino acids, mainly due to the activity of microbial decarboxylases. Fish products are specially concerned by the presence of biogenic amines as they have high amino acid content, and are contaminated by decarboxylase positive bacterial flora. In cold smoked salmon, histamine, tyramine, cadaverine and putrescine are the main biogenic amines that are produced by the bacterial flora. (Jorgensen and others 2000b). Whereas the production of histamine, cadaverine and putrescine can be attributed to Gram negative spoilage flora e.g. *Photobacterium phosphoreum* or enterobacteria like *Morganella morganii* or *Serratia* sp. (Kim and others 2000; Emborg and others 2002), tyramine production on fish products is mainly the consequence of the presence and growth of *Photobacterium phosphoreum*, and also lactic acid bacteria from the genus *Carnobacterium* and *Lactobacillus* (Jorgensen and others 2000; Emborg and others 2002). Among lactic acid bacteria, the genus *Carnobacterium* is well represented at the end of the shelf-life of cold smoked salmon (Leroi and others 1998; Gonzalez-Rodriguez and others 2002). Many carnobacteria strains are known for bacteriocin production, and this property could be exploited on such lightly preserved product to control the development of food pathogens like *Listeria monocytogenes.* We have isolated three strains, *Carnobacterium divergens* V41, *Carnobacterium piscicola* V1 (Pilet and others 1995) and SF668 (Duffes and others 1999a) that have been shown to reduce the growth of inoculated *Listeria monocytogenes* in sterile cold-smoked salmon (Brillet and others 2004) without affecting the sensory attributes of the product (Brillet and others 2005). These strains could be used as a biopreservative agents to control *Listeria monocytogenes* risk in cold smoked salmon. However, production of tyramine ranging from 35 µg/g to 122 µg/g was observed on cold smoked salmon for the three strains (Brillet and others 2005). These levels are on the range of naturally contaminated cold smoked salmon that frequently contain more than 200 µg/g of tyramine at the end of the storage (Jorgensen and others 2000a; Connil and others 2002b). Tyramine can have toxicological effects on sensitive consumers when high amounts are produced in food (Ten Brink and others 1990; Santos 1996) and although it has never been implicated in fish poisoning, the production of this biogenic amine by a strain used for biopreservation could be a barrier to its use in the european legislative context (Wessels and others 2004). For that reason, we have performed in this study the screening of natural tyramine negative strains among the genus *Carnobacterium* and the isolation and first characterization of a mutant strain from *Carnobacterium divergens* V41 that produces no tyramine.

2. Material and methods

Bacterial strains and media

Carnobacterium divergens V41, *Carnobacterium piscicola* V1 and SF668 were isolated from trout intestine and cold smoked salmon (Pilet and others 1995; Duffes and others 1999a). *Enterococcus faecalis* JH2-2 was obtained from Université de Caen, and *Listeria innocua* 1 from the ENITIAA collection.
A collection of 35 *Carnobacterium* strains isolated from cold smoked salmon was supplied by IFREMER. *Listeria monocytogenes* strains were isolated from French salmon smoked plants and were supplied by ASEPT (Laval, France). All the strains were propagated in Elliker broth at 30°C for carnobacteria and *Enterococcus,* at 37°C for *Listeria* strains.

Screening of tyramine non producing strains by PCR- based test

Two PCR primers TDC1 (5'-GAAGCACAAATTCGTCATTTA-3') and TDC2 (5'-TAACCAATCATTTTTTTCCAT-3') were designed from the alignment of sequences of available bacterial tyrosine decarboxylase genes (program MULTALIN

http://prodes.toulouse.inra.fr/multalin/; Corpet 1988). DNA was extracted from *Carnobacterium* strains as described by Rachman and others (2004). The amplification of potential *tdc* gene were performed in a total volume of 20 µl containing 1X PCR buffer, 0.2 mM dNTPs, 0.4 µM of each primer, 1.5 mM $MgCl_2$, 1 U Taq polymerase and 40 ng of bacterial DNA. PCRs were carried out in a PT-100 thermocycler (MJ Research, Bio-Rad, Waltham, USA) with a first denaturation step of 7 min at 94°C, followed by 35 cycles of 1 min 94°C, 45 s at 49°C, 4 min at 72°C and a final extension step of 7 min at 72°C. Amplified products were analysed by electrophoresis in a 3% (wt/v) agarose gel in Tris-acetate-EDTA buffer and were subsequently visualized by UV illumination after ethidium bromide staining.

Mutagenesis and isolation of mutants

The experimental procedure used in this work was described previously (Richard and others 2003). Briefly, 10 to 120 µl of ethyl methyl sulfonate (Sigma M0880, d = 1,7 g/ml) was added to 2 ml of exponential growth cultures of *Carnobacterium divergens* V41 and incubated at 30°C for 2 h. After centrifugation, the cells were washed twice with physiological water and appropriated dilutions were plated onto Elliker agar plates and incubated 48h at 30°C. Colonies obtained from culture with less than 10% survival rate were picked and studied for tyramine production.

Tyramine production detection and quantification

The colonies obtained from the mutagenesis procedure were inoculated in microplates containing 150 µl modified Maijala broth (Tryptone 5 g/L, yeast extract 4 g/L, Meat extract 8 g/L, tyrosine 2 g/L, Tween 80 0,5 g/L, MgSO4 0,02 g/L, CaCO3 0,01 g/L, MnSO4 0,005 g/L, FeSO4 0,004 g/L, bromocresol purple 0,006 g/L) (Maijala 1993). After incubation at 30°C for 48-72h, apparition of violet colour suggesting alcalinisation of the media indicated tyramine producing strains. All the isolates giving no alcalinisation were selected for tyramine quantification on HPLC by the method described previously (Connil and others 2002a). *Enterococcus faecalis* JH2-2 and *Listeria innocua* 1 were used respectively as tyramine producer and tyramine non producer controls.

Characterization of the mutant

The tyrosine decarboxylase negative strain was characterized in comparison with the wild strain by fermentation profile using API 50 CH (Biomérieux, Marcy l'Etoile, France). Antibiotic resistance was checked on wild and mutant strains using 18 antibiotics (Biomérieux, Marcy l'Etoile, France) reported in table 1 by the disc diffusion test. Bacteriocin activity was tested as follow : *Carnobacterium divergens* V41 and A8 were grown at 30°C for 24 h in Elliker broth. After centrifugation (8000 rpm, 6 min.), cell-free supernatant (CFS) was treated 10 min at 80°C. CFS was serially two-fold diluted in phosphate buffer (0,1 M, pH 6.5) and 10 µl of each dilution was spotted onto Elliker agar plates containing 10^7 CFU/ml of *Listeria innocua* 1 indicator strain. The bacteriocin activity was defined as the reciprocal of the lowest dilution showing no inhibition of the indicator strain in arbitrary units AU/ml. Bacteriocin spectrum on 57 *Listeria monocytogenes* strains described in a previous study (Brillet and others 2004) were checked by spotting filtered supernatant of *C. divergens* V41 and A8 onto Elliker agar plates containing 10^7 CFU/ml of each *Listeria* strain.

Growth on sterile cold-smoked salmon and tyramine production

Sterile cold-smoked salmon (CSS) was prepared as described by Joffraud and others (1998). Each *Carnobacterium* strain was inoculated in CSS as described before (Brillet and others 2004). Briefly, appropriate dilutions of each *Carnobacterium* strain were mixed and inoculated (2 % v/w) in parts of 30 g of thawed sterile CSS pieces distributed in 15 polyamide polyethylene bags (Bourdeau, Saint Etienne de Montluc, France). Pieces were gently mixed with the inoculating solution and samples were then vacuum-packed and incubated for 28 days using the following conditions : 9 days at 4°C followed by 19 days at 8°C, with a break

during 2 h at 20°C after 19 days of storage (industrial recommendations NF V 01-003, 2004). Microbial analysis was done weekly in triplicates in Elliker plates incubated aerobically for 5 days at 20°C. Results are expressed as mean of three measures ± 95% Confidence Interval (CI = 1.96 x $\sqrt{\text{standard deviation}^2/3}$).

Tyramine was determined by HPLC on salmon flesh as described before (Brillet and others 2005) after 14 and 28 days of storage.

3. Results and discussion

Screening of tyrosine decarboxylase negative *Carnobacterium* strains by PCR

A collection of 35 *Carnobacterium* strains isolated from cold smoked salmon was checked for the presence of potential *tdc* gene by PCR. A fragment of 300 bp (fig. 1) corresponding to the amplification using specific *tdc* primers was obtained for all the *Carnobacterium* strains. All the strains were also detected as tyramine positive by alcalinisation of modified Maijala's broth. These results suggest that all the strains are potential tyramine producers. To confirm this hypothesis, tyramine production measured by HPLC on 10 strains chosen randomly among the 35 showed that the strains produced around 1500 µg/ml tyramine after 24 to 48h of growth in Maijala's medium. These results showed that strains from the genus *Carnobacterium* isolated from cold smoked salmon are tyramine producers as it has been shown previously for carnobacteria isolated from meat products (Masson and others 1996). As no natural tyramine negative mutants could be isolated, the strategy of mutagenesis was chosen for further studies.

Isolation of tyramine – mutants from *Carnobacterium divergens* V41

5300 colonies of *Carnobacterium divergens* V41 were screened on modified Maijala's broth after exposure to ethyl methyl sulfonate (EMS). Twelve colonies showing no alcalinisation of the media were streaked on Elliker agar and re-inoculated in modified Maijala to check tyramine production. One isolate showing no tyramine production was selected and named V41A8. Tyramine quantification on HPLC was checked for this mutant strain and the wild strain after 1 and 5 days of incubation at 30°C. Tyramine production was around 2051 µg/ml for the wild strains after 1 days and 2069 µg/ml after 5 days, whereas no tyramine production was detectable for V41A8. This strain was thus kept for the following experiments.

After PCR amplification of the potential *tdc* gene with the specific primers used before, a positive result was therefore obtained for V41 and V41A8 (data not shown). The mutation has probably affected separated bases on the tyrosine decarboxylase gene of V41A8. In this case, the annealing of the primers remains possible leading to a positive result for the mutant strain. The sequencing of the whole tyrosine decarboxylase gene of V41 and V41A8 would confirm this hypothesis. This result demonstrated the limits of the PCR method for the screening of tyramine positive strains. Positive PCR results should then be confirmed with tyramine detection or quantification as it was done in this study with the *Carnobacterium* strain collection.

The screening of 5300 colonies was necessary for the isolation of one stable mutant after EMS exposition, and this rate was in agreement with the study of Joosten who used the same method on *Enterococcus faecalis* (Joosten 1995). The mutant V41A8 was considered as stable as no revertant could be detected after several propagation of the strain.

Characterization of the mutant

The fermentation profile determined on 50 carbohydrates was identical between both strains : glycerol, ribose, D-glucose, D-fructose, D-mannose, N-acetylglucosamine, amygdaline, arbutine, esculine, salicine, cellobiose, maltose, sucrose, trehalose, b-gentiobiose, and

gluconate were positive, all the other sugars were not fermented. Both strains showed also the same antibiotic resistance and sensitivity (Table 2). These results suggest that the mutation procedure did not affect the main metabolic activities.

The comparison of inhibition properties of both strains is also important for the characterization of the tyramine negative strain. The wild strain *Carnobacterium divergens* V41 produces a bacteriocin named divercin V41 that is active against *Listeria monocytogenes* (Pilet and others 1995; Metivier and others 1998). This bacteriocin is involved in the inhibition effect of the strain against *Listeria monocytogenes* in cold smoked salmon (Duffes and others 1999b; Richard and others 2003; Brillet and others 2004). Bacteriocin activity was 12800 AU/ml on *Listeria innocua* 1 for both mutant and wild strains showing that the mutation did not affect bacteriocin production. Moreover, study of the inhibition spectrum against a large number of *Listeria monocytogenes* strains showed that both strains gave inhibition zones on all the 57 strains tested. All these results show that the tyramine negative strains have kept all its inhibition properties in liquid media.

Growth on sterile cold-smoked salmon and tyramine production

The growth of both strains during storage is shown on Fig. 2. For the wild strain, the growth began at 4°C, from $1.2 \pm 1.1 \times 10^5$ CFU/g to $4.3 \pm 1.2 \times 10^7$ CFU/g after 4 weeks of storage. The mutant strain grew more slowly than the wild strain during the three first weeks of storage but reached the similar level of $1.6 \pm 1.2 \times 10^7$ CFU/g after the four weeks. It is possible that the mutation procedure had affected growth adaptability of the strain at low temperatures or on fish muscle that is mainly composed of amino acids, peptides and proteins.

A small amount of tyramine (43 µg/g) was detected after 14 days when the product was inoculated with the strain *C. divergens* V41 and it increased to 122 µg/g after 28 days of storage (Table 2). This production could be directly attributed to the strain as no tyramine was detected at all on the uninoculated control. On the opposite, when the mutant strain V41A8 was inoculated on the product, no tyramine could be detected after 14 days nor after 28 days of storage.

4. Conclusion

In conclusion of this study, we have shown that tyramine production is a characteristic of *Carnobacterium* strains isolated from cold smoked salmon. However, it is possible to obtain by a simple chemical mutation a tyramine negative strain that keeps interesting inhibition properties. The strain is able to grow on cold smoked salmon at refrigerated temperatures without producing any detectable amount of tyramine. Further studies are needed to determine the effect of this strain on the inhibition of *Listeria monocytogenes* in cold smoked salmon and on the quality of the product. As it has been obtained by simple mutagenesis without recombinant DNA, this strain is not considered as a genetic modified organism (GMO) as defined in the Directive 2001/18/EC (2001). However, the use of bacterial strains as *Carnobacterium divergens* V41 or V41A8 for the biopreservation of non fermented foods will require complete informations about their taxonomy, safety and consumer perception (Wessels and others, 2004).

Acknowledgments

This work was supported by grants from the “Aliment Qualité Sécurité” project no. R01 / 05 from the French Ministry of Agriculture and Fishery, and from partners CITPPM, ENITIAA, IFREMER and ASEPT.

References

Brillet A, Pilet MF, Prevost H, Bouttefroy A, Leroi F. 2004. Biodiversity of *Listeria monocytogenes* sensitivity to bacteriocin-producing *Carnobacterium* strains application in sterile cold-smoked salmon. J Appl Microbiol 97:1029-1037.

Brillet A, Pilet MF, Prévost H, Cardinal M, Leroi F. 2005. Effect of inoculation of *Carnobacterium divergens* V41, a biopreservative strain against *Listeria monocytogenes* risk, on the microbiological, chemical and sensory quality of cold-smoked salmon. Int J Food Microbiol 104:309-324.

Connil N, Le Breton Y, Dousset X, Auffray Y, Rince A, Prevost H. 2002a. Identification of the *Enterococcus faecalis* tyrosine decarboxylase operon involved in tyramine production. Appl Environ Microbiol 68:3537-3544.

Connil N, Prevost H, Dousset X. 2002b. Production of biogenic amines and divercin V41 in cold smoked salmon inoculated with *Carnobacterium divergens* V41, and specific detection of this strain by multiplex-PCR. J Appl Microbiol 92:611-617.

Corpet F. 1988. Multiple sequence alignment with hierarchical clustering. Nucl Ac Res 16:10881-10890.

Directive 2001/18/EC of the European parliament and of the council of 12 March 2001 on the deliberated release into the environment of genetically modified organisms. Official Journal of the European Communities, 17/04/2001.

Duffes F, Leroi F, Boyaval P, Dousset X. 1999a. Inhibition of *Listeria monocytogenes* by *Carnobacterium* spp. strains in a simulated cold smoked fish system stored at 4 degrees C. Int J Food Microbiol 47:33-42.

Duffes F, Corre C, Leroi F, Dousset X, Boyaval P. 1999b. Inhibition of *Listeria monocytogenes* by in situ produced and semipurified bacteriocins of *Carnobacterium* spp. on vacuum-packed, refrigerated cold-smoked salmon. J Food Prot 62:1394-1403.

Emborg J, Laursen BG, Rathjen T, Dalgaard P. 2002. Microbial spoilage and formation of biogenic amines in fresh and thawed modified atmosphere-packed salmon (*Salmo salar*) at 2 degrees C. J Appl Microbiol 92:790-799.

Gonzalez-Rodriguez MN, Sanz JJ, Santos JA, Otero A, Garcia-Lopez ML. 2002. Numbers and types of microorganisms in vacuum-packed cold-smoked freshwater fish at the retail level. Int J Food Microbiol 77:161-168.

Joffraud J.J, Leroi F, Chevalier F. 1998. Development of a sterile cold-smoked fish model. J Appl Microbiol 85:991-998.

Joosten HM. 1995. Isolation of tyrosine decarboxylaseless mutants of a bacteriocin-producing *Enterococcus faecalis* strain and their application in cheese. J Food Prot 58:1222-1226.

Jorgensen LV, Dalgaard P, Huss HH. 2000a. Multiple compound quality index for cold-smoked salmon (Salmo salar) developed by multivariate regression of biogenic amines and pH. J Agric Food Chem 48:2448-2453.

Jorgensen LV, Huss HH, Dalgaard P. 2000b. The effect of biogenic amine production by single bacterial cultures and metabiosis on cold-smoked salmon. J Appl Microbiol 89:920-934.

Kim SH, Ben-Gigirey B, Barros-Velazquez J, Price RJ, An H. 2000. Histamine and biogenic amine production by *Morganella morganii* isolated from temperature-abused albacore. J Food Prot 63:244-251.

Leroi F, Joffraud JJ, Chevalier F, Cardinal M. 1998. Study of the microbial ecology of cold-smoked salmon during storage at 8 degrees C. Int J Food Microbiol 39:111-121.

Maijala RL. 1993. Formation of histamine and tyramine by some lactic bacteria in MRS-broth and modified decarboxylation agar. Lett Appl Microbiol 17:40-43.

Masson F, Talon R, Montel MC. 1996. Histamine and tyramine production by bacteria from meat products. Int J Food Microbiol 32:199-207.

Metivier A, Pilet MF, Dousset X, Sorokine O, Anglade P, Zagorec M, Piard JC, Marion D, Cenatiempo Y, Fremaux C. 1998. Divercin V41, a new bacteriocin with two disulphide bonds

produced by *Carnobacterium divergens* V41: primary structure and genomic organization. Microbiol 144:2837-2844.
Pilet MF, Dousset X, Barré R, Novel G, Desmazeaud M, Piard JC. 1995. Evidence for two bacteriocins produced by *Carnobacterium piscicola* and *Carnobacterium divergens* isolated from fish and active against *Listeria monocytogenes*. J Food Prot 58:256-262.
Rachman C, Kabadjova P, Valcheva R, Prévost H, Dousset X. 2004. Identification of *Carnobacterium* species by restriction fragment lenght polymorphism of the 16S – 23S rRNA gene intergenic spacer region and species-specific PCR. Appl Env Microbiol 70:4468-4477.
Richard C, Brillet A, Pilet MF, Prevost H, Drider D. 2003. Evidence on inhibition of *Listeria monocytogenes* by divercin V41 action. Lett Appl Microbiol 36:288-292.
Santos MHS. 1996. Biogenic amines : their importance in foods. Int J Food Microbiol 29:213-231.
Ten Brink B, Damink C, Joosten HM, Huis in 't Veld JH. 1990. Occurrence and formation of biologically active amines in foods. Int J Food Microbiol 11:73-84.
Wessels S, Axelsson L, Hansen EB, De Vuyst L, Laulund S, Lahteenmaki L, Lindgren S, Mollet B, Salminen S, von Wright A. 2004. The lactic acid bacteria, the food chain, and their regulation. Trends Food Sc Technol 15:498-505.

Table 1 Antibiogram spectrum of *C. divergens* V41 and V41A8. R : resistant ; S : sensitive ; I : intermediate.

Antibiotic	Resistance or sensitivity of *C. divergens* V41 and V41A8
Pénicillin G	R
Ampicillin	S
Cefalotin	I
Cefotaxim	R
Streptomycin	R
Gentamicin	R
Kanamycin	R
Chloramphénic	S
Tétracyclin	S
Erythromycin	S
Spiramycin	S
Colistin	R
Vancomycin	S
Triméthoprim	S
Nalidixic acid	R
Norfloxacin	I
Rifampicin	S
Fusidic acid	I

Table 2 Tyramine production in sterile batches of cold-smoked salmon in the presence of *C. divergens* V41 or *C. divergens* V41A8, stored under vacuum during nine days at 4°C and 19 days at 8°C. Each result represents the mean of three measures (95% confidence interval).

		Tyramine µg g^{-1}
Control (sterile CSS)	D14	nd*
	D28	nd*
C. divergens V41	D14	43.0 (± 23.2)
	D28	122.4 (± 9.4)
C. divergens V41A8	D14	nd*
	D28	nd*

*nd : not detected

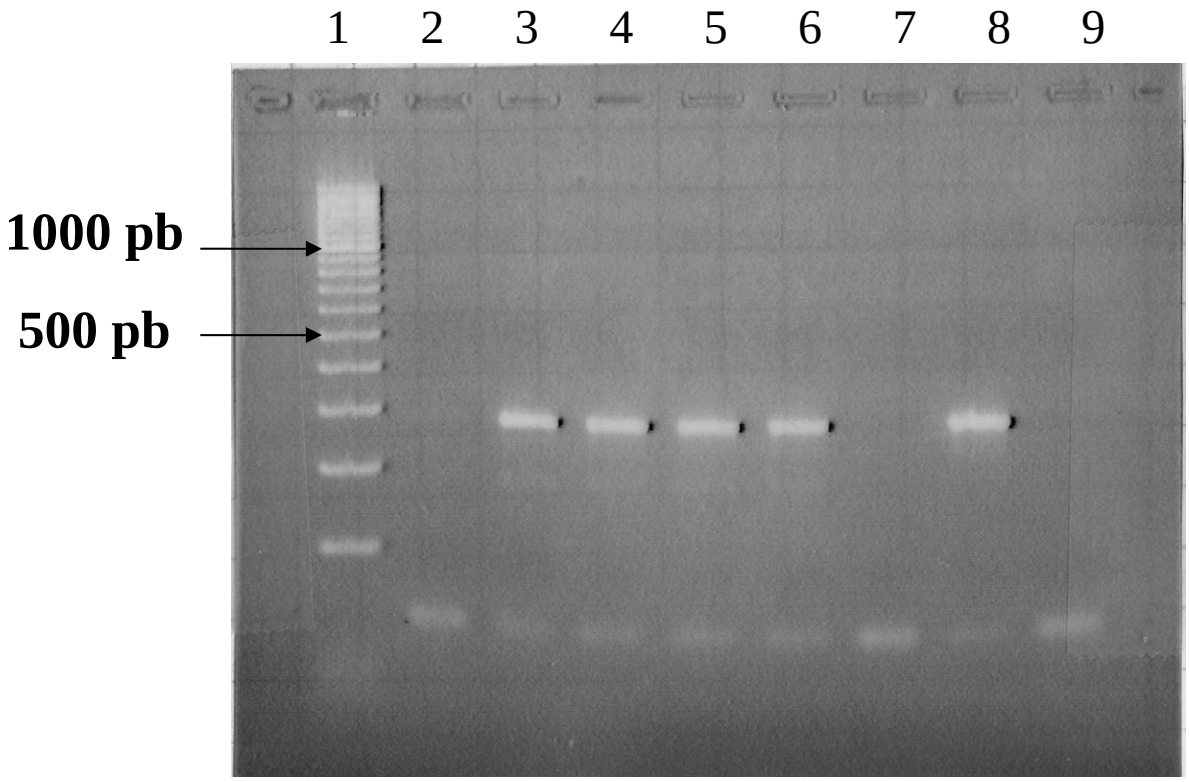

Figure 1 : amplification product of potential *tdc* gene on *Carnobacterium* strains. 1 : 100 bp molecular weight ladder (Biolabs), 2 and 7 : tyramine negative lactic acid bacteria strains, 3 to 6 and 8 : *Carnobacterium* SF2025, SF2051, SF2052, SF2053, SF2055 from the collection. 9 : negative control.

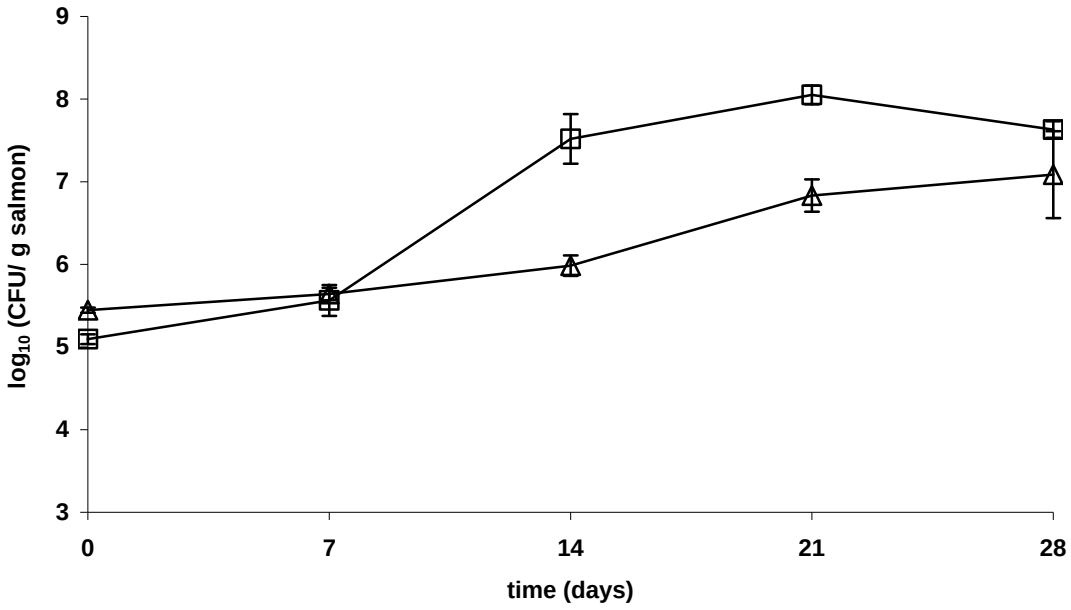

Figure 2 Growth of *C.divergens* V41 (□) or V41A8 (△)inoculated in sterile cold smoked salmon during storage (9 days at 4°C and 19 days at 8°C with a break of 2h at 20°C after 19 days). Bars indicate 95% confidence intervals.