Efficiency of enzymes and benzalkonium chloride treatments against *Listeria monocytogenes* dual-species biofilms determined by fluorescence microscopy and image analysis

Rodríguez-López, P., Nimo, V., Carrera-Iglesias, A., Blanco. T and Cabo, M.L.*

Marine Research Institute (IIM-CSIC), Vigo (Pontevedra), Spain *Corresponding author: marta@iim.csic.es

BACKGROUND

Persistence of *Listeria monocytogenes* in food-related environments has been considered of a great concern over the last decade (1, 2). It forms part of multispecies biofilm communities (3) which confers *L. monocytogenes* and accompanying microbiota higher resistance to traditional treatments based on quaternary ammonium compounds (4).

OBJECTIVE

To assess the **EFFECTIVENESS** of sequentially combined application of **CELLULASE (CEL)** or **PRONASE (PRO)** with **BENZALKONIUM CHLORIDE (BAC)** against the elimination of 168h - *L. monocytogenes* dual species biofilms potentially present in fish and dairy industries.

MATERIALS AND METHODS

1. BACTERIAL CONSORTIA

Previously isolated from surfaces of food related premises.

Fish industry consortium		Dairy industry consortium	
L. monocytogenes A1	*E. coli* A 14	*L. monocytogenes* G1	*P. fluorescens* B52

2. DUAL-SPECIES BIOFILM SET UP

10^7 – 10^8 CFU/ml of each species were mixed 1:1 and cultured in Tryptic Soy Broth supplemented with 0.6 g/l yeast extract and 2.5 g/l D-glucose on 10 x 10 x 1 mm AISI 316 stainless steel coupons put in 24 well-plates at 25ºC and 100 rpm in saturated humidity conditions.

3. EXPERIMENTAL DESIGN

According to a first order factorial experimental design,enzymatic treatments were applied followed by a solution of benzalkonium chloride on coupons at concentrations detailed in Tables 1 and 2. Samples were stained using LIVE/DEAD BacLight® Bacterial Viability Kit (Life Technologies) and efficiency of treatments was assessed by epifluorescence microscopy followed by image analysis with Metamorph MM AF (Molecular Devices).

Encoded values	PRN (UI/l)	BAC (ppm)
(0,0)	3850	1025
(-1,-1)	700	50
(1,1)	7000	2000
(-1,1)	700	2000
(1,-1)	7000	50

Encoded values	CEL (UI/l)	BAC (ppm)
(0,0)	0.11	1025
(-1,-1)	0.02	50
(1,1)	0.2	2000
(-1,1)	0.02	2000
(1,-1)	0.2	50

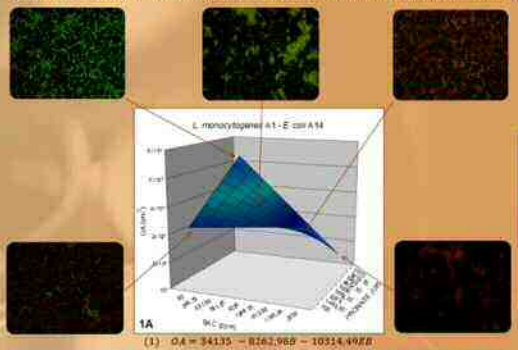
Tables 1 and 2: Concentrations of PRN, CEL and BAC used.

Additionally, in order to avoid ambiguities among the effects and also to identify specific dispersion effects of the treatments tested, detached viable cultivable cells were also quantified in every experimental set performed.

RESULTS AND DISCUSSION

Obtained results were significantly described by empirical equations [1-4] and permits to deduce the following **GENERAL** and **SPECIFIC** effects:

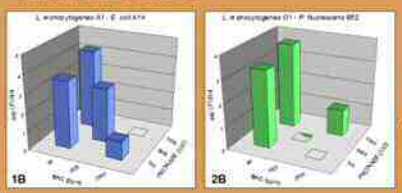
- **Occupied area (OA) by living cells (Figures 1A to 4A):** a synergic effect between PRN and BAC in decreasing occupied area was demonstrated in fish and dairy consortia. However, sequential application of CEL and BAC even has counter-productive effects against the occupied area by the biofilms. So, BAC antimicrobial activity seems to be somehow annulled by cellulose. Moreover, according to equations there is a slight individual effect of cellulose in decreasing the occupied in the fish consortium was detected, though not significantly lower compared with PRN. This is in contrast with previous results that demonstrated cellulase and other polisaccharidases have anti-biofilm properties (5, 6). Effectiveness of PRN as a mixture of proteolytic enzymes could be also reflected in the biofilm's architecture probably due to biofilms' matrix composition which in case of *Listeria monocytogenes* is mainly of a proteic nature (7). This variation is especially noticeable in the fish consortium where the uniformity of the structure is lost, forming cellular aggregates, as the concentration of PRN rises. Thus, it could be hypothesized presence of PRN changes the biofilm structure from uniformity to aggregates and this permits BAC to reach more number of bacterial cells, thus giving rise to the observed synergic effect.Regarding biofilm sensibility to treatments, fish consortium is more resistant to PRN – BAC treatment that dairy consortium. In fact, besides OA values were close to zero in most of the experimental ambit, maximum values of OA reached were significantly lower (250 µm²) when comparing with those obtained in the fish consortium (around 55000 µm²).
- **Detached living cells (Figures 1B to 4B):** BAC clearly decreases spread viable cultivable cells released from the biofilm, probably as a consequence of its effect as disinfectant. In regard to the enzymes, higher dispersing capacity was demonstrated in the case of cellulase comparing with pronase at high BAC concentrations, probably related with the detected synergy in decreasing the occupied area in this last.

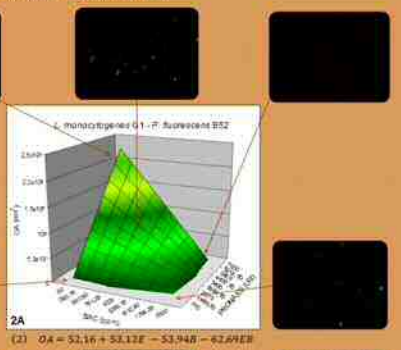


(1) $OA = 34135 - 8262{,}98B - 10314{,}49EB$

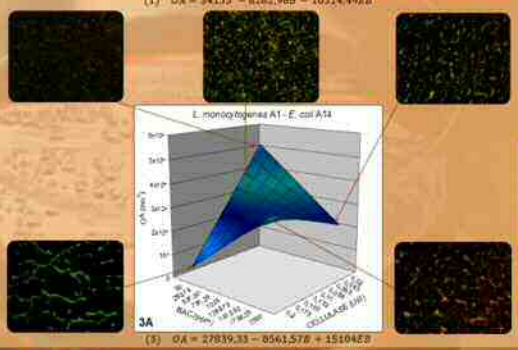
Figure 1: Effect of PRN-BAC treatments on *L. monocytogenes* – *E.coli* biofilms. (A) Occupied area by living cells and (B) detached living cells after treatment.

Figure 2: Effect of PRN-BAC treatments on *L. monocytogenes* – *P. fluorescens* biofilms. (A) Occupied area by living cells and (B) detached living cells after treatment.





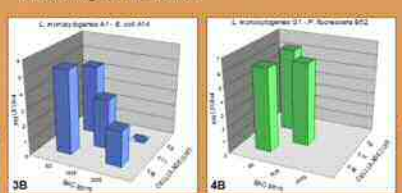
(2) $OA = 52{,}16 + 53{,}12E - 53{,}94B - 62{,}69EB$

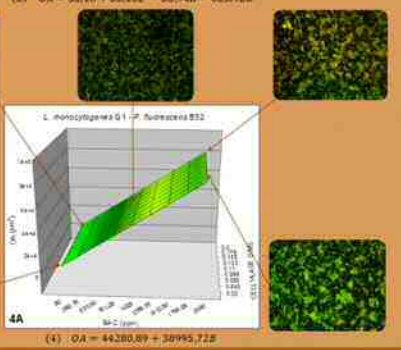


(3) $OA = 27839{,}33 - 8561{,}57B + 15104EB$

Figure 3: Effect of CEL-BAC treatments on *L. monocytogenes* – *E.coli* biofilms. (A) Occupied area by living cells and (B) detached living cells after treatment.

Figure 4: Effect of CEL-BAC treatments on *L. monocytogenes* – *P. fluorescens* biofilms. (A) Occupied area by living cells and (B) detached living cells after treatment.





(4) $OA = 44280{,}89 + 38995{,}72B$

CONCLUSIONS

1. PRN – BAC treatments decreases the total occupied area by live cells of mature dual-species biofilms formed by *Listeria monocytogenes* and *E. coli* or *P. fluorescens*. Although antibacterial features of the treatment are more likely to be due to the effect of BAC,PRN appears to act synergically dislodging biofilms' structure and thus allowing BAC to penetrate easier.
2. No significant positive effect was found when BAC was combined with CEL. Simultaneous action of these elements promotes an increase of OA values, thus making it inappropriate to be used in real industrial settings
3. To avoid inefficient disinfections, detached living cells should be considered when designing *in situ* cleaning and disinfection procedures since the propagation of living cells could lead to uncontrolled pathogen spread into different premises increasing the risk of transmission to final food products.

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