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Short communication

Effect of high hydrostatic pressure and activated film packaging on bacterial diversity of fruit puree

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ABSTRACT

The aim of the present study was to determine the effect of high hydrostatic pressure (HP) and films activated with a combination of enterocin AS-48 and thymol (AF) on the microbial load and bacterial diversity of fruit puree (banana, apple, pear). HP and AF were applied singly, or in combination (AFHP). Samples were chill-stored. HP and AFHP treatments reduced viable counts significantly ($P \leq 0.05$). The predominant bacterial groups in the puree were fam. *Comamonadaceae* (23.17%) and genera *Methylobacterium* (21.46%), *Acidovorax* (8.70%) and *Sphingomonas* (6.63%). *Pseudomonas* became relevant by the end of storage (10.50%). Most of the AF samples had higher relative abundances of *Comamonadaceae, Methylobacterium, Acidovorax* and *Sphingomonas* and lower relative abundance of *Pseudomonas*. Application of HP treatment markedly reduced the relative abundances of *Comamonadaceae, Methylobacterium, Acidovorax* and *Sphingomonas*. *Lactobacillales* increased in relative abundance upon application of HP treatment but not during storage, while *Pseudomonas* increased towards the end of storage. The combined treatment (AFHP) achieved greatest reduction in the relative abundances of *Comamonadaceae* and *Methylobacterium*. Results indicate that AFHP treatment had greatest effects on the bacterial diversity of the fruit puree and was also the most effective in keeping total aerobic mesophiles and Enterobacteriaceae below detectable levels.

1. Introduction

Fresh fruit purees are attractive food products for infant feeding, but also for the elderly and for people in general interested in consumption of ready-to-eat foods rich in fibre, vitamins, antioxidants and other bioactive compounds. However, they may contain a variety of spoilage microorganisms and foodborne pathogens (Akhbariyoon, Mirbagheri, & Emtiazi, 2016; Yeni, Yavaş, Alpas, & Soyer, 2016). In order to ensure food preservation and safety and to satisfy consumer preferences for fresh-tasting fruits and purees with high nutritional and sensory qualities, the demand for alternative non-thermal preservation methods has increased.

High-pressure (HP) processing technology is widely applied for preservation of vegetable foods (Wang, Huang, Hsu, & Yang, 2016). HP treatment shows less impact than thermal processing on low molecular weight compounds such as vitamins and pigments (Khosravi-Darani, 2010; Khosravi-Darani & Vasheghani-Farahani, 2005; Wang et al., 2016). The efficacy of HP treatments can be improved in combination with other hurdles such as natural antimicrobial compounds (Patterson, 2005). Among them, bacteriocins and essential oils and their phenolic compounds are the best studied. Thymol is one of the major constituents of thyme oil (Hyldgaard, Mygind, & Meyer, 2012) and is a cheap antimicrobial commercially available. Enterocin AS-48 is a circular bacteriocin that has shown promising results as a preservative in vegetable foods (Grande-Burgos, Pérez-Pulido, López-Aguayo, Gálvez, & Lucas, 2014).

Deciphering the effects of HP treatments combined with natural antimicrobials on the bacterial populations in foods could help to better understand their efficacy in food preservation. High-throughput sequencing technology has become a powerful tool for studying microbial communities in food systems (Kergourlay, Taminiau, Daube, & Champomier Vergès, 2015). Yet, there is no information on how non-thermal treatments affect the microbiota of fresh fruit purees. The aim of the present study was to determine the bacterial diversity of a fruit puree stored under refrigeration and the effects on the microbiota of a mild HP treatment applied singly or in combination with an activated film packaging containing thymol and enterocin AS-48.

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Table 1
Total aerobic mesophiles from controls and treated fruit puree samples at different storage times under refrigeration.

	Storage time (days)			
	0	2	7	10
Control	3.25 ± 0.21[a]	3.77 ± 0.09[a]	4.38 ± 0.15[a]	2.69 ± 0.13[a]
Activated film (AF)	2.95 ± 0.07[a]	3.80 ± 0.31[a]	2.60 ± 0.17[b]	1.48 ± 0.08[b]
HP	< 1.0[b]	1.75 ± 0.29[b]	1.25 ± 0.08[b]	< 1.0[b]
AFHP	< 1.0[b]	1.05 ± 0.07[b]	< 1.0[b]	< 1.0[b]

Data (log_{10} colony-forming units, CFU/g) are the average of two replicates ± standard deviation.
[a, b] down columns indicate significantly different ($P \leq 0.05$) values.

2. Materials and methods

2.1. Sample preparation and treatments

Fruit puree was prepared right before use from pears, bananas and Golden delicious apples as described in a previous study (Ortega Blázquez, Grande Burgos, Pérez-Pulido, Gálvez, & Lucas, 2017). The pH of the puree was determined with a pH meter (Crison, Barcelona, Spain).

Polyethylene–polyamide bags (10 × 15 cm) were activated with antimicrobials by addition of 1 ml 0.25% thymol (Sigma, Madrid, Spain) plus 1 ml of partially-purified enterocin AS-48 (0.5 mg/ml) as described elsewhere (Ortega Blázquez, Grande Burgos, Pérez-Pulido, Gálvez, & Lucas, 2018). Bags not activated with antimicrobials were used as controls.

Aliquots (5 g) of the puree were packed inside the bags, followed by sealing under vacuum. Two replicates (each one in triplicate) were prepared for each experimental condition. Controls (C) consisted of puree packed in bags not activated with antimicrobials. Activated film samples (AF) consisted of puree packed in bags activated with enterocin AS-48 and thymol. High-hydrostatic pressure treatment (300 MPa for 5 min at room temperature) was applied to samples packed in bags without antimicrobials (HP) and also to samples packed in activated bags (AFHP) as described elsewhere (Ortega Blázquez et al., 2018). All samples were stored for 10 days at 5 °C. At desired incubation times, triplicate samples from each treatment condition (C, AF, HP, AFHP) were homogenized with 10 ml sterile saline solution, serially-diluted and plated in triplicate on trypticase soya agar (TSA; Scharlab, Barcelona, Spain). Viable cell counts were determined after 24–48 h incubation at 37 °C.

In separate experiments, three independent batches of purees were treated by HP and AFHP as above and stored for 20 days at 5 °C, and periodically inspected for viable counts of total aerobic mesophiles (TSA), Enterobacteriaceae (McConkey agar, Scharlab) and *Listeria* (Palcam agar with added supplement, Schalab).

Table 2
Effect of treatments on viable counts from three batches (A, B, C) of fruit puree during refrigerated storage.

	Batch	Storage time (days)				
		0	2	5	10	20
Control (TAM)	A	3.73 ± 0.15[a]	3.58 ± 0.20[a]	3.61 ± 0.08[a]	4.94 ± 0.23[a]	6.48 ± 0.34[a]
	B	3.64 ± 0.09[a]	3.48 ± 0.24[a]	3.60 ± 0.07[a]	4.53 ± 0.18[a]	6.43 ± 0.27[a]
	C	3.38 ± 0.24[a]	3.81 ± 0.17[a]	3.43 ± 0.18[a]	4.72 ± 0.29[a]	6.41 ± 0.29[a]
Control (EB)	A	1.78 ± 0.16[a]	1.49 ± 0.05[a]	2.87 ± 0.29[a]	4.08 ± 0.35[a]	5.93 ± 0.42[a]
	B	1.00 ± 0.11[a]	1.00 ± 0.12[a]	1.30 ± 0.21[a]	4.23 ± 0.27[a]	5.32 ± 0.34[a]
	C	1.52 ± 0.14[a]	1.45 ± 0.13[a]	2.45 ± 0.16[a]	4.15 ± 0.23[a]	3.20 ± 0.27[a]
Control (L)	A	< 1.00[a]	< 1.00[a]	< 1.00[a]	< 1.00[a]	< 1.00[a]
	B	< 1.00[a]	< 1.00[a]	< 1.00[a]	< 1.00[a]	< 1.00[a]
	C	< 1.00[a]	< 1.00[a]	< 1.00[a]	< 1.00[a]	< 1.00[a]
Control (pH)	A	4.16	4.14	4.10	4.01	3.32
	B	4.15	4.14	4.11	4.03	3.19
	C	4.00	4.04	4.00	3.00	2.83
HP (TAM)	A	1.84 ± 0.09[b]	1.90 ± 0.16[b]	1.84 ± 0.17[b]	1.90 ± 0.23[b]	3.33 ± 0.27[b]
	B	1.30 ± 0.12[b]	1.30 ± 0.08[b]	1.30 ± 0.10[b]	1.30 ± 0.17[b]	3.37 ± 0.19[b]
	C	1.48 ± 0.07[b]	< 1.00[b]	< 1.00[b]	1.00 ± 0.09[b]	3.13 ± 0.21[b]
HP (EB)	A	1.79 ± 0.17[b]	< 1.00[b]	< 1.00[b]	< 1.00[b]	1.60 ± 0.14[b]
	B	1.00 ± 0.07[b]	< 1.00[b]	< 1.00[b]	< 1.00[b]	2.41 ± 0.23[b]
	C	< 1.00[b]	< 1.00[b]	< 1.00[b]	< 1.00[b]	2.20 ± 0.27[b]
HP (L)	A	< 1.00[a]	< 1.00[a]	< 1.00[a]	< 1.00[a]	< 1.00[a]
	B	< 1.00[a]	< 1.00[a]	< 1.00[a]	< 1.00[a]	< 1.00[a]
	C	< 1.00[a]	< 1.00[a]	< 1.00[a]	< 1.00[a]	< 1.00[a]
HP (pH)	A	4.16	4.10	4.08	4.0	4.02
	B	4.12	4.12	4.10	4.06	4.06
	C	4.06	4.00	3.99	3.90	3.96
AFHP (TAM)	A	1.04 ± 0.07[b]	1.10 ± 0.09[b]	< 1.00[b]	1.27 ± 0.12[b]	2.83 ± 0.18[b]
	B	< 1.00[b]	< 1.00[b]	< 1.00[b]	1.00 ± 0.07[b]	2.27 ± 0.31[b]
	C	< 1.00[b]	< 1.00[b]	< 1.00[b]	< 1.00[b]	2.43 ± 0.23[b]
AFHP (EB)	A	< 1.00[b]	< 1.00[b]	< 1.00[b]	< 1.00[b]	< 1.00[b]
	B	< 1.00[b]	< 1.00[b]	< 1.00[b]	< 1.00[b]	1.28 ± 0.27[b]
	C	< 1.00[b]	< 1.00[b]	< 1.00[b]	< 1.00[b]	1.14 ± 0.33[b]
AFHP (L)	A	< 1.00[a]	< 1.00[a]	< 1.00[a]	< 1.00[a]	< 1.00[a]
	B	< 1.00[a]	< 1.00[a]	< 1.00[a]	< 1.00[a]	< 1.00[a]
	C	< 1.00[a]	< 1.00[a]	< 1.00[a]	< 1.00[a]	< 1.00[a]
AFHP (pH)	A	4.15	4.12	4.11	4.05	4.02
	B	4.14	4.11	4.09	4.02	4.04
	C	4.07	4.05	3.89	4.00	3.97

Controls and samples treated by high pressure alone (HP) or in combination with the activated film (AFHP) were inspected for viable counts: total aerobic mesophiles (TAM), Enterobacteriaceae (EB) and Listeria (L).
Data (log_{10} colony-forming units, CFU/g) are the average of two replicates ± standard deviation.
[a, b] down columns indicate significantly different ($P \leq 0.05$) values between controls and treated samples.

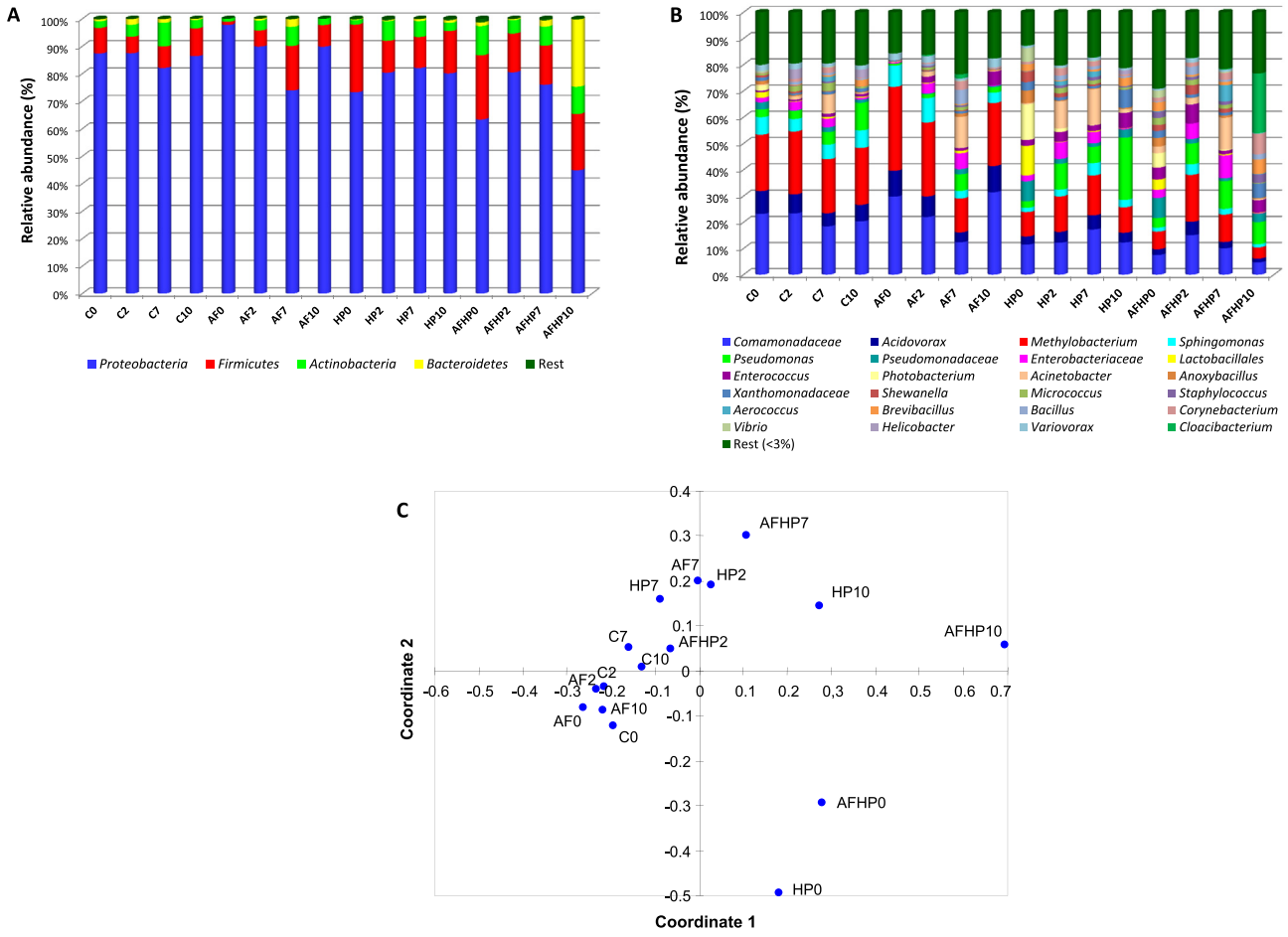

Fig. 1. Relative abundance of operational taxonomic units (OTUs) in refrigerated fruit puree samples. OTUs were clustered at phylum (**A**) or genus (**B**) level. C, controls packed in films without antimicrobials. AF, samples packed in films activated with thymol plus enterocin AS-48. HP, samples packed in films without antimicrobials and treated by high-hydrostatic pressure. AFHP, samples packed in films activated with thymol and enterocin AS-48 and treated by high-hydrostatic pressure. Sampling was performed at days 0, 2, 7 and 10. Panel **C**, Principal Coordinates analysis (PCoA).

2.2. DNA extraction

Bacterial DNA was prepared as described elsewhere (Ortega Blázquez et al., 2018). The DNA from the two batch replicates and same sampling point was pooled into a single sample and its concentration and quality was measured (NanoDrop spectrophotometer; Thermo Scientific, United Kingdom).

2.3. DNA sequencing and analysis

Illumina technology was applied to obtain 16S rRNA gene sequences as described elsewhere (Caporaso et al., 2011; Ortega Blázquez et al., 2018). The following primers were used to amplify the V3 and V4 regions of the 16S gene (Klindworth et al., 2013): TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG (forward) and GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC (reverse). Amplification, sequence analysis and assignment of reads to operational taxonomic units (OTUs) were carried out as described elsewhere (Ortega Blázquez et al., 2018).

2.4. Statistical analysis

Data corresponding to viable cell counts were analysed by one-way ANOVA (Microsoft Excel 2007). Paleontological Statistics (PAST) was used to calculate diversity indices Shannon Wiever (H'), Simpson (D) and Chao 1. Euclidean distance matrix was obtained with SPSS software.

3. Results and discussion

Counts for total aerobic mesophiles (TAM) in the fruit puree (pH 4.48) only increased significantly ($P \leq 0.05$) by storage day 7, but then decreased at day 10 (Table 1), possibly due to the lower pH (3.87). TAM from samples packed in activated bags (AF) only were significantly lower ($P \leq 0.05$) than controls at days 7 and 10, possibly because of delayed diffusion of antimicrobials into the food matrix added to the decrease in pH (4.0). HP reduced TAM below detectable levels (< 1 log CFU/g) right after treatment, followed by an increase in counts at days 2 and 7 that could possibly be due to recovery of sublethally-injured cells. The final pH of HP (4.27) and AFHP samples (4.40) had not decreased markedly. The combined treatment AFHP was the most effective for keeping viable counts below detection level during storage.

In separate experiments, three batches of fruit puree packed in bags activated or not with antimicrobials were treated by HP and stored for 20 days (Table 2). TAM and Enterobacteriaceae increased in all control samples during storage. In this second experiment, acidification did not take place until day 20 (except batch C), indicating batch to batch variations both in bacterial counts and pH, especially during late storage. *Listeria* was not detected either in controls or in the treated samples. Enterobacteriaceae were below detectable levels in HP samples from days 2–10 and in AFHP samples from days 0–10. However, none of the treated samples were protected from growth of

Enterobacteriaceae by day 20.

The number of operational taxonomic units (OTUs) obtained after high-throughput sequencing for puree samples stored for 10 days ranged from 38681 to 128099 (Supplementary Table 1). *Proteobacteria* were the predominant group in the fruit puree, exceeding 60% in relative abundance for most of the samples, except for the combined treatment at day 10 (Fig. 1A). *Firmicutes* were the second most abundant group, and showed higher relative abundances in several treated samples (e.g., AF7, 16.26%; HP0, 24.67%; HP10, 15.43%; AFHP0, 23.38%; AFHP10, 20.49%). *Actinobacteria* also showed higher relative abundances in HP-treated samples. *Bacteroidetes* showed much lower relative abundances, except for AFHP10.

Control samples (C0) showed highest relative abundance for fam. *Comamonadaceae* (23.17%), followed by *Methylobacterium* (21.46%), *Acidovorax* (8.70%) and *Sphingomonas* (6.63%) (Fig. 1B). The relative abundance of these bacterial groups remained quite stable during storage of the control samples, except for the increase in OTUs assigned to *Pseudomonas* at the end of storage (10.50%). *Comamonadaceae* is a diverse bacterial group (Willems, 2014) that includes genus *Acidovorax*, also detected in the study. It is tempting to suggest that the OTUs not assigned to genus but included in Fam. *Comamonadaceae* could also belong to *Acidovorax*. *Acidovorax* was reported in the microbiota of diseased soil from banana farms (Xue et al., 2015) and from the interior of banana plant pseudostem (Rossmanna et al., 2012). Therefore we could speculate that bananas could be the primary source for *Acidovorax* in the puree. *Methylobacterium* spp. was detected in apple phyllosphere (Yashiro & McManus, 2012) and pseudostems of banana plants (Suhaimi et al., 2017). This is the first report on *Methylobacterium* as a relevant bacterial group in fruit puree. Altogether, results from the present study suggest that the main representatives in the microbiota of the puree are plant-associated bacteria.

The bacterial diversity from purees packed in the activated films resembled that of control samples, except that *Comamonadaceae* and *Methylobacterium* had higher relative abundances for most of the sampling points. Application of the HP treatment markedly reduced the relative abundances of *Comamonadaceae* (11.48%), *Methylobacterium* (9.29%), *Acidovorax* (3.13%) and *Sphingomonas* (1.67%) while other groups (*Lactobacillales*, *Photobacterium*, *Pseudomonadaceae*) increased. During storage, *Pseudomonas* increased to 23.68% (HP10).

The combined treatment (AFHP0) achieved a still greater reduction in the relative abundances of *Comamonadaceae* (7.50%) and *Methylobacterium* (6.67%). These results suggest that inactivation by HP was enhanced by the antimicrobials from the activated film. Nevertheless, *Pseudomonas* did not seem to be affected by treatments and reached 10.55% at day 7. Furthermore, *Cloacibacterium* reached a high relative abundance during late storage (22.76% in AFHP10), being the main representative of *Bacteroidetes*. *Cloacibacterium* has been described previously in wastewaters and in human skin (Gay, Fleming, & Oh, 2016), but not in vegetable foods.

Principal Coordinates Analysis (Fig. 1C) revealed that most of the controls and activated film samples clustered closely (except AF7), while the HP-treated samples were more dispersed. The most distantly-related samples were from HP treatment singly or in combination with activated film at time 0 (HP0, AFHP0) and end of storage (HP10, AFHP10).

In conclusion, the combined AFHP treatment was the most effective in keeping viable counts below detection levels during storage and also induced the greatest changes in the bacterial diversity of the purees.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2018.10.083.

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