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Enhancing high throughput sequencing unveils changes in bacterial communities during ready-to-eat lettuce spoilage

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ABSTRACT

Purpose: Spoilage flora is critical in vegetal ready to eat (RTE) product shelf-life and selecting efficient spoilage control technologies depends on the microorganisms present. This manuscript describes the evolution of the bacteriome of Batavia RTE lettuce, from fresh lettuce up to completely spoiled (day 14) and correlate these results with the sensorial characteristics. **Research Method:** The microbiome of vegetal RTE were examined using culture-dependent and culture-independent (16S rRNA metabarcoding) methods. Culture-dependent methods were related with the metagenomic results and sensory analysis to describe the evolution during spoilage and shelf-life. **Findings:** Our results demonstrated that the RTE lettuce bacteriome during spoilage is dominated by Gram-negative bacteria, mainly *Flavobacterium* and *Pseudomonas*. A bacterial population of 22 operational taxonomic units (OTUs) represent up to 96% of total bacterial reads and is maintained during the spoilage, representing the bacterial core of RTE lettuce. A high correlation was detected between culture-independent and culture-dependent results, both in general and selective culture media. Sensorial analysis of lettuce demonstrated that "odor" was the key parameter to determine the sensorial spoilage time and is related to total microbial load and to high concentrations of spoilage-related bacterial genera. **Limitations:** Hereby presented results are limited by the lettuce variety and by the storage conditions (MAP, 6°C, up to 14 days). **Originality/Value:** This paper describes an overview of the microbial and sensory evolution during spoilage of Batavia lettuce under MAP. A combination of culture-dependent and independent methods and sensorial analysis were used up to 14 days of storage.

INTRODUCTION

Minimally processed vegetables are appealing to consumers because they represent a good compromise between healthy food and convenience. Consumer acceptability and likeness of ready to eat (RTE) fresh-cut fruit and vegetable products are measured as a combination of sensorial attributes, such as visual appearance, texture and flavor, as well as a combination of nutritional and safety aspects. In this regard, the maintenance of these features for a longer time has a critical impact on producers' economic benefits by reducing costs while ensuring consumer satisfaction. The unavoidable spoilage of vegetables with time is characterized by brown discoloration, necrosis, loss of texture, exudation and/or production of off-flavors (Ponce et al., 2002). Most studies linked sensory rejection of RTE vegetables point to an increased microbial load (Jacxsens et al., 2003; Ragaert et al., 2006).

The bacterial community associated with plant tissues (phyllosphere), is a complex ecosystem, were human pathogens and no pathogens can survive and/or grow. The microbiological studies on RTE vegetable products have focused on food security, although no pathogens contribute greatly to sensory impairment. However, the so-called specific spoilage organisms (SSO) are responsible for generating off-odors and flavors, shortening the life of these products. These SSO have been less studied, despite the knowledge of who they are and how they behave is crucial in order to develop efficient conservation and packing strategies.

The number and type of microorganisms found on fresh products are highly variable and dependent on product type, agronomic practices, weather conditions as well as the harvest, transportation and further processing and handling (Ahvenainen, 1996; Olaimat & Holley, 2012). Gram-negative bacteria dominate the microflora associated with most vegetables (Tournas, 2005). The microbiota of vegetables and fruits is made up largely of *Pseudomonas spp*., *Erwinia herbicola*, *Flavobacterium*, *Xanthomonas*, and *Enterobacter*. Lactic acid bacteria, such as *Leuconostoc mesenteroides* and *Lactobacillus spp*., are also commonly found. Finally, yeasts such as *Torulopsis*, *Saccharomyces* and *Candida* as well as various molds (like *Alternaria*, *Penicillium*, *Fusarium* and *Aspergillus*) are part of the dominant microorganisms, mostly in fruits due to their high sugar content (Caponigro et al., 2010; De Azeredo et al., 2011).

The application of molecular techniques in the microbial ecology of food has changed the way of studying the microbial diversity in complex food ecosystems, including vegetables (Abriouel et al., 2008). As a result, molecular approaches have been successfully applied to describe the bacterial ecosystem in (RTE) vegetables, or even to detect viruses in lettuce leaves (Aw et al., 2016). Most of these studies have monitored the microbial communities in lettuce, focusing on the initial bacteriome or on the differences between cultivation season, cultivation characteristics of lettuce variety (Allende et al., 2004; Caponigro et al., 2010; Oliveira et al., 2010; Salgado et al., 2014). New molecular identification techniques in microbial ecology have revealed new dominant species in plant products, such as *Oxalobacter* or *Flavobacterium* in RTE salads, while new microbial species have been described in RTE spinach (Lopez-Velasco et al., 2011). Comparing the results obtained by different research groups, lettuce variety seems to be a critical factor in bacterial composition. For example, Rudi et al. (2002) compared the dominant microorganisms in Norwegian and Spanish RTE lettuces and concluded that *Pseudomonas* was the dominant group in Norwegian lettuce and *Enterobacteriaceae* was dominant in the Spanish ones.

Batavia lettuce (*Lactuca sativa*, L), also known as summer crisp lettuce, is the most common lettuce variety in the north area of Spain, and it is widely used for RTE salads. The aim of this work was to monitor the changes in RTE Batavia lettuce quality and to

characterize the microbial ecosystem during refrigerated storage with a combination of complementary methods, sensory, conventional microbiology and molecular analysis, in order to better understand the spoilage process in this important commercial product. A deep characterization of bacteriome is the base of further works trending to increase the product's shelf-life.

MATERIAL AND METHODS

Product characterization

Batavia lettuce was produced and packaged (400 ± 15 g) under a modified atmosphere (MAP 100% N_2) by a local manufacturer (Spain). Samples were stored at 4 ± 0.5 ºC for 24 hours and transported to the laboratory under refrigerated conditions (day 1) and subsequently incubated at 6 ± 0.5 ºC in a climatic chamber up to 14 days. Commercial shelf-life was eight days according to the manufacturer.

At each sampling time (1, 2, 4, 7, 9, 11, 14 days), 10 g of lettuce were aseptically transferred to stomacher bags, 90 mL of peptone water with added NaCl (APT, 0.5% NaCl w/v, Pronadisa, Spain) and it was homogenized for 1 min in a Stomacher (Seward Stomacher Lab-Blender 400, Seward Medical, London, UK). Appropriate decimal dilutions from the homogenized were prepared in APT and plated to enumerate the following microorganisms: (i) total viable bacteria (TVC) were enumerated in plate count agar (PCA, Pronadisa, Spain) incubated for 7 days at 10ºC; (ii) *Enterobacteriaceae* were enumerated in violet red bile glucose agar (VRBGA, Pronadisa, Spain) incubated aerobically at 37ºC for 1 day; (iii) Lactic acid bacteria were enumerated on MRS (Pronadisa, Spain) for 2-3 days at 30ºC under anaerobic conditions; (iv) *Pseudomonas* were counted in *Pseudomonas* agar (CM0559B with SR103 supplement, both from Oxoid, UK) and incubated for 1-2 days at 25ºC; (v) Yeast were enumerated in Saboureau agar (Pronadisa, Spain) supplemented with chloramphenicol (0.1g.l^{-1}) after 48 hours at 25ºC. At each sampling time, 3 independent lettuce-bags were used as independent replicates. All analyses were done in duplicate for each independent replicate. The bags were disposed after each sampling day.

The CO_2 and O_2 concentrations in the headspace of RTE lettuce bag were determined in duplicate at each sampling time using a PBI Dansensor Checkmate 9900 gas analyzer (Ringsted, Denmark). Additionally, lettuce cleaning water (inlet) was aseptically sampled in the production facilities at the packaging day. One hundred (100) ml of this sample was filtered (Microplus 21 STL system, 0.45 µm pore, Whatman) and filter was used for the same microbiological analysis described for lettuce.

DNA extraction, and amplicon library generation and sequencing

In each sampling day, 15 ml of the samples prepared as abovementioned (1/10 in APT) were spin down at 3600 g for 10 minutes at 4 ºC, the supernatant was removed and the pellet stored at -20 ºC up to DNA extraction for no more than 15 days). The DNA was extracted using Wizard® SV Genomic DNA Purification System (Promega, USA) with minor modifications. Briefly, bacterial pellet and 0.5 g of glass-beads were diluted in 600 µl of lysis solution and beadbeated (6 x 20 sec on and 10 sec off; Disruptor genie, Scientific industries inc., USA). Bacterial debris were spin down (30 sec, 3000 g), the pellet was discarded and the purification continued as proposed by the manufacturer. Finally, DNA was diluted in 50 µl of sterile MilliQ water and DNA concentration was quantified using Nanodrop spectrophotometer (Nanodrop Technologies, USA).

Amplicon libraries were prepared using the primers Gray28F (5'-TTGATCNTGGCTCAG-3') and Rev338 (5'-TGCCTGCCTCCCGTGGAGT-3'), which

target the V1-V2 region of the small subunit ribosomal RNA (16S rRNA) gene (Huse et al., 2008). These primers are appropriate for 454 sequencing following manufacturers specifications for Lib-L preparation. For each reaction, 20-40 ng of DNA was mixed with 25 µl of 2X DNA polymerase master mix red (Ampliqon, USA), 1µM of each primer solution and MilliQ water up to 50 µl. The DNA was subsequently amplified under the following conditions: 10 min at 98 ºC, 30 cycles of 10 s at 98 ºC, 30 s at 53 ºC and 30 sec at 72 ºC, and a final step of 10 min at 72 ºC. The PCR product length was verified using gel electrophoresis with 0.8% agarose in TAE buffer at 70 v (Biorad, Madrid, Spain) and visualised in a Biodoc-it imaging system (UVP, Upland, USA). PCR products were purified using Illustra GFX PCR DNA and Gel Band Purification Kits (GE Healthcare, Spain) following the recommended protocol. DNA concentration was quantified using a nanodrop spectrophotometer (Nanodrop Technologies, USA) and equimolar amounts of each PCR product were mixed in a pool. DNA was amplified by Emulsion-PCR before sequencing on 2 lanes of a 4-lane PicoTiterPlate (PTP). Pyrosequencing was performed with the Genome Sequencer (GS) FLX (Roche-454 Life Science) according to GS FLX Titanium (454-Roche Life Science) method manuals provided by Roche/using the massively parallel pyrosequencing protocol (Margulies et al., 2005) in the CITIUS Biology Service (University of Sevilla, Sevilla, Spain).

Sequence analysis

Amplicon sequence analyses were performed using the Mothur 454 SOP (Schloss et al., 2009; 2011). Briefly, sequences were demultiplexed allowing 1 and 2 mismatches for the index and primer respectively and those shorter than 200 nucleotides were removed. Finally, the reads were aligned against the SILVA v128 (Quast et al., 2013) database with subsequent chimera and chloroplast and mitochondrial sequences removal. Only samples with > 1000 reads were retained for taxonomic analysis.

Sensory analysis

Sensory analysis was performed by six to eight previously trained panellists from the staff at the Food Research Division, AZTI, to evaluate the freshness of the lettuce. The panellists undertook five training sessions, each lasting 1 h, using a predefined glossary of attributes and three review sessions were performed before the start of the experiment. RTE lettuce was evaluated as described by ISO standard 4121:198721 using a five-point descriptive scale, where 1 is absolutely fresh, 3 is the rejection limit and 5 is completely spoiled. The following attributes of RTE lettuce were assessed: Texture, Flavour, Bright, Odour and Colour homogeneity. Lettuce was defined as unacceptable when the score of any sensory attribute was equal to or more than 3.

Statistical analysis

The software PSPP V0.6.2 (Free Software Foundation, Inc.) was used in order to perform the analysis of variance (ANOVA) and the least significant difference (LSD) statistical procedures for sensory and culture-dependent analysis. A confidence interval of 95% ($p \leq 0.05$) was used.

RESULTS AND DISCUSSION

Sensorial analysis and atmosphere evolution

Sensory analysis of the samples determined a shelf life of the product between 7 and 9 days, mainly due to sensorial defects in odour (Table 1), while the texture was the parameter with the lowest change during the storage time. These results agree with previously published

studies. Allende et al. (2004) studied "Lolo rosso " RTE lettuce and reported that aroma was the parameter with the lowest score at the end of shelf life, while the study of Ioannidis et al. (2018) with iceberg lettuce stored in anaerobic MA highlighted the odour as the critical rejection parameter after 6.6 storage days. The apparition of theses off-odours has been related with lettuce metabolism, microbial metabolism or a combination of both parameters (Ioannidis et al., 2018).

Oxygen concentration in the bags was reduced from 2% at day 1 to close to 0% after 4 days, without a significant increase along storage (Fig. 1). In contrast, CO_2 increased during the storage up to 11% in 11 days (Fig. 1). According to the bibliography, CO_2 increase during storage is usual in RTE vegetables (Rudi et al., 2002; Allende et al., 2004; Ares et al., 2008; Ioannidis et al., 2018) as a consequence of vegetal-tissue respiratory metabolism.

Culture dependent results

In the inlet cleaning water, microbiological counts in all considered agars were below the detection limit (10 cfu.ml^{-1}), demonstrating that water was not a contamination source. Cleaning water is intensively chlorate at inlet point to reduce the flora and can represent a selective factor in fresh lettuce.

Table 1. Evolution of sensorial scores along the storage

	Parameters				
Time (Days)	Texture	Flavour	Bright	Odour	Colour
1	8.2	8.1	6.9	7.0	8.2
2	7.0	7.3	9.1	7.1	7.3
4	6.5	6.6	5.1	5.9	6.7
7	6.5	5.7	5.4	5.4	5.6
9	6.0	4.7	4.5	4.5	4.7
11	4.1	4.0	3.3	3.0	3.3
14	2.0	0.6	3.0	0.6	1.6

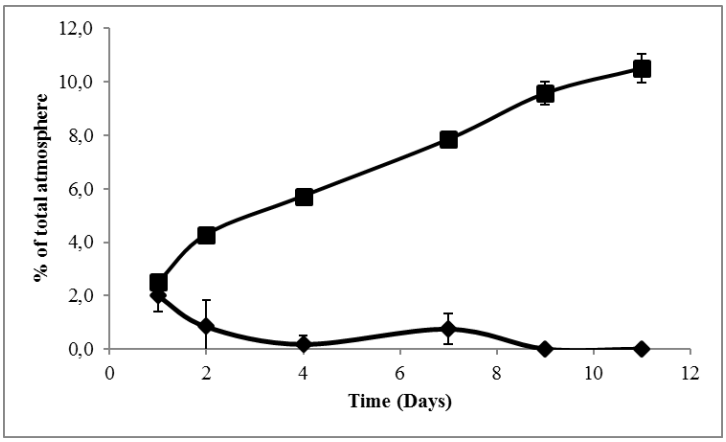

Fig. 1. Gas (O_2 and CO_2) composition (%) during the storage. No represented (up to 100%) was N_2. (■CO_2 concentration; ♦O_2 concentration).

The total bacterial load was around 5.5 Log cfu.g^{-1} at day 1 and increased above 9.8 Log cfu.g^{-1} after 14 days (Table 2). Previous studies indicate a variation in mesophilic bacteria concentration after harvest from 3 to 9 Log cfu.g^{-1} in raw vegetables, depending on the productive and growing conditions (Zagory, 1999; Oliveira et al., 2010; Yu et al., 2018). Bacterial load close to 5.6 Log cfu.g^{-1} has been described in lettuce (Hunter et al., 2010) and in Batavia lettuce variety (Di Carli et al., 2016). An evolution of total bacteria load during storage below 6 ºC from 4 up to 7-8 Log cfu.g^{-1} was observed in fresh lettuce (Allende et al., 2004) and RTE salades (Rudi et al., 2002). In Iceberg lettuce, Bercardino et al. (2018) found an average of 8.5 Log cfu.g^{-1} at middle shelf-life, while Ioannidis et al. (2018) referred 8.3 Log cfu.g^{-1} after 10 days. Data obtained hereby agree with the model proposed by Tsironi et al. (2017).

The selective agars analysis showed that counts in Pseudomonas agar and TVC were similar along the spoilage time (from 10^5 to over 10^9 cfu.g^{-1}, Table 2), suggesting that *Pseudomonas* is one of the mayor genus in lettuce during spoilage. Similar results were obtained in RTE vegetables by other researchers (Rudi et al., 2002; Legnani & Leoni, 2004; Ioannidis et al., 2018).

Initial counts of LAB and *Enterobacteriaceae* were below 4 Log cfu.g^{-1} (Table 2), and both groups would represent below 5 % of total flora in fresh lettuce. Similar initial levels have been described in iceberg lettuce (Ioannidis et al., 2018). While the RTE lettuce became spoiled, counts growth up to 8.6 Log cfu.g^{-1} in VRBG and 6.5 in MRS. Counts on selective media are, usually, less than 10 % to the total counts, but this depends on the considered vegetable. Hereby described results agree with the general metabolic characteristics of both groups (LAB and *Enterobacteriace*ae), those can grow easily even under refrigeration and/or in the absence of oxygen. Along with storage, LAB counts were, approximately, 3 magnitude orders lower than the TVC counts at end of commercial life. A similar situation has been described in the bibliography with RTE lettuce stored in MAP at 5 ºC (Allende et al., 2004; Ioannidis et al., 2018).

Finally, yeast and mould counts started at 4.3 cfu.g^{-1} and increased up to 7.8 cfu.g^{-1} after 11 days (Table 2). These data are lower compared to the ones reported by Rudi et al. (2002) after 10 to 12 days of storage and by Ioannidis et al. (2018) for 10 days.

Bacterial community changes during spoilage time

A total of 286,782 raw reads were obtained, from which 114,277 remained after quality control and chloroplast and mitochondrial sequences removal. The average number of raw reads per sample was 11,950, which corresponds to 4,762 quality filtered bacterial reads (Table 3). Interestingly, although the number of raw reads per sample was not statistically related to sampling time, the proportion of bacterial reads increased with time, as does the number of bacterial OTUs (Fig. 2), suggesting an increased bacterial load and diversity in samples collected later in time. Sample 7A had a considerably lower number of valid reads than the corresponding replicates and was removed from further interpretations.

During the spoilage, *Bacteriodetes* and *Proteobacteria* were the main phyla presented in the RTE lettuce, with more than 90% of the total bacteria identified. *Firmicuttes* is a non-dominant phylum, in contrast with the results described by Yu et al. (2018) in lettuce from North Korea. Differences in origin or in the lettuce variety (not described in their work) would explain these differences. During the spoilage, 709 different OTU (Operational Taxonomic Units) had been identified, and, as observed in Table 3, the number of OTU per sample increased along the spoilage time, up to the day 14. Statistical differences ($p<0.05$) were detected in the number of OTU during spoilage (Fig. 2), indicating higher bacterial diversity in late spoiled samples. Initial OTU richness results seem in agreement with the ones

described before for organic and conventional growth lettuce (Leff & Fierer, 2013) and, on the other hand, the increase in bacterial richness in spoiled salads has been described recently (Di Carli et al., 2016).

In the present work, 56 families and 105 different genera were identified. Our data demonstrate that the bacteriome of RTE Batavia lettuce is dominated by Gram-negative bacteria, mainly *Pseudomonas*, *Flavobacterium*, *Chyseobacterium* and bacteria from the "Enteric_bacteria_cluster" (Table 4). These genera summed around 90% of the total identified sequences in each sample. Sequence analysis showed that all samples from day 4 until day 14 shared 22 OTUs from 8 genera: *Flavobacterium* (10 OTUs), *Chryseobacterium* (4 OTUs), Enteric_bacteria_cluster and *Pseudomonas* (2 OTUs each) and *Janthinobacterium*, *Acidovorax*, *Carnobacterium* and *Duganella* (1 OTU each). These OTUs represented between 75% and 98% of the bacteria detected in each sample when spoilage is detectable (Fig. 3, starting at day 4) and confirmed the stability of the bacterial population present in RTE lettuce duringthe spoilage. The idea of a stable bacterial core in vegetal phyllosphere have been introduced by Rastogi et al. (2012) and seem to be confirmed by the results obtained in the present work since data obtained at day 1 and 2 (fresh lettuce, Table 3) indicated that most of these OTUs are also present at end of storage time.

The class *Flavobacteriales* (including *Flavobacterium* and *Chryseobacterium*) was found the major class present in RTE lettuce during the spoilage (Table 4). *Flavobacterium* has been isolated in different ecosystems, including soil, water and food products. However, their importance during food spoilage is still unclear. *Flavobacterium* genus presented high diversity, with 3 main OTUs but hundreds of OTUs with lower proportional importance. This genus represents up to 64.2±13.1 % of total bacteriome on day 7, although its relative importance decreased after this time. The ability to grow at low temperature and oxygen concentration (Alfaro et al., 2013) would explain the initial proliferation phase. Conversely, other authors have identified *Flavobacterium* in RTE lettuce at lower levels than those described hereby (1% of total bacteria) (Rastogi et al., 2012) or do not describe this genus as important in fresh harvested lettuce (Yu et al., 2018), suggesting that storage conditions would facilitate *Flavobacterium* proliferation.

Table 2. Bacterial counts (Log cfu.g^{-1}) in RTE lettuce along spoilage in different cultivation mediums

Time (Days)	VRBG	*Pseudomonas*	TVC	MRS	Yeast
1	3.8 ± 0.2^{a}	5.2 ± 0.5^{a}	5.5 ± 0.7^{a}	3.6 ± 0.0^{a}	4.3 ± 0.3^{a}
2	4.3 ± 0.5^{ab}	5.4 ± 0.2^{a}	6.0 ± 0.3^{a}	3.7 ± 0.3^{a}	4.8 ± 0.2^{a,b}
4	5.1 ± 0.5^{b}	6.8 ± 0.2^{b}	7.3 ± 0.2^{b}	4.1 ± 0.5^{a}	5.7 ± 0.9^{b}
7	6.7 ± 0.3^{c}	8.1 ± 0.2^{c}	8.3 ± 0.2^{c}	5.4 ± 0.2^{b}	6.8 ± 0.2^{b,c}
9	7.8 ± 0.2^{d}	8.5 ± 0.0^{c}	8.9 ± 0.2^{d}	6.2 ± 0.8^{b,c}	7.1 ± 0.0^{c}
11	8.2 ± 0.6^{d,e}	9.4 ± 0.1^{d}	9.5 ± 0.1^{e}	6.2 ± 0.7^{b,c}	7.8 ± 0.2^{c}
14	8.6 ± 0.1^{e}	9.5 ± 0.3^{d}	9.8 ± 0.2^{e}	6.5 ± 0.2^{c}	7.3 ± 0.4^{c}

Data are average of 3 independent samples.
^{a,b,c,d,e,} different letters in the same columns indicate significant differences ($p<0.05$).

Table 3. Number of reads obtained per sample (raw) and those remaining after quality filtering (QC filter) and after chloroplast and mitochondrial read removal (Bacterial). Number of OTUs identified per sample

Sample ID	Number of reads			Number of OTUs
	Raw	QC filter	Bacterial	
1A	5019	4998	185	27
1B	14949	14934	73	14
1C	18168	18146	70	14
2A	8516	8475	311	24
2B	16068	16035	337	22
2C	13362	13333	187	23
4A	15264	14391	8479	109
4B	19331	19082	1179	52
4C	14683	14467	1777	56
7A	57	56	35	na
7B	3263	3109	1965	31
7C	9426	8965	3502	56
9A	11829	10877	8109	83
9B	16286	15128	11694	78
9C	6674	5345	4347	72
11A	10316	8961	7244	69
11B	16873	15166	12589	93
11C	12536	10685	9555	112
14A	11210	10044	8066	95
14B	16893	14999	10920	83
14C	14072	11653	8706	83
Total	286782	263779	114277	708

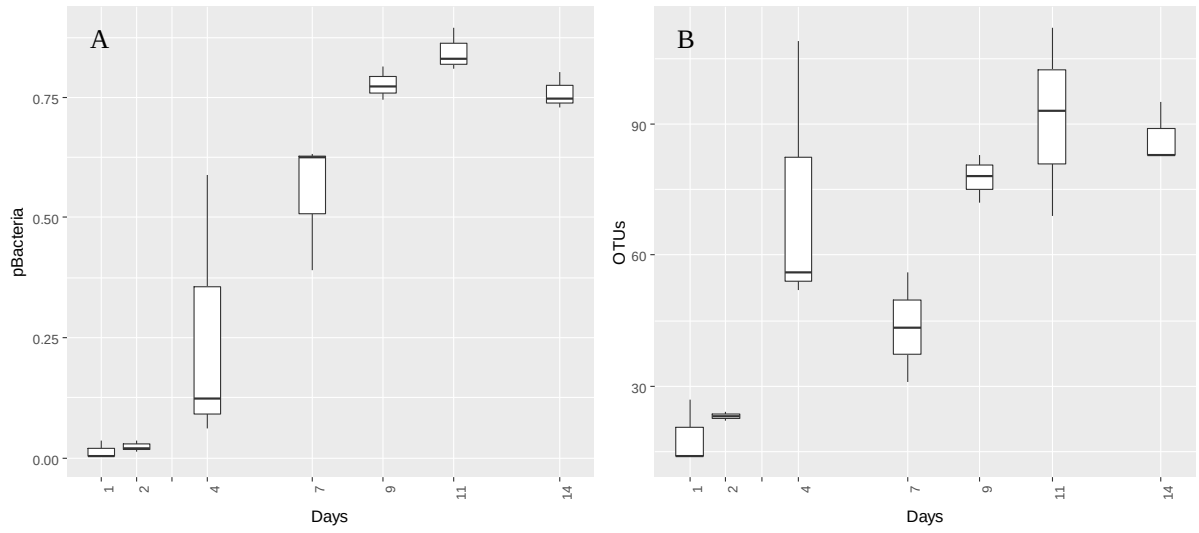

Fig. 2. Proportion of bacterial reads (considering all three replicates) from the total quality filtered reads per sampling time (A) and the total number of OTUs found per sampling time (B).

Table 4. Bacteriome (as % of total bacteria) of RTE lettuce during spoilage up to 14 days

	Storage time (days)						
Genus	1	2	4	7	9	11	14
Flavobacterium	39.1±9.7	42.0±19.4	56.4±17.2	68.3±10.3	64.7±9.9	56.8±10.3	57.2±3.0
Pseudomonas	12.8±2.2	19.4±8.4	24.4±12.3	16.9±3.4	15.9±2.5	18.3±4.8	21.6±5.3
Janthinobacterium	2.5±0.9	2.6±2.3	5.3±0.6	6.3±2.3	9.4±6.3	8.9±2.2	10.4±2.2
Enteric_Bacteria_cluster	1.0±1.6	0.7±0.8	1.7±1.4	2.6±2.3	3.5±1.6	4.9±5.9	2.5±0.4
Chryseobacterium	5.1±3.6	3.7±2.0	4.0±2.9	1.7±0.2	1.3±0.5	2.3±2.2	1.4±0.7
LAB	6.0±7.0	2.3±3.7	1.1±1.0	0.8±0.5	0.8±1.0	1.0±1.3	1.3±0.3
Others	33.6±2.5	29.3±25.7	7.0±1.7	3.3±1.6	4.4±1.7	7.9±6.1	5.7±2.1

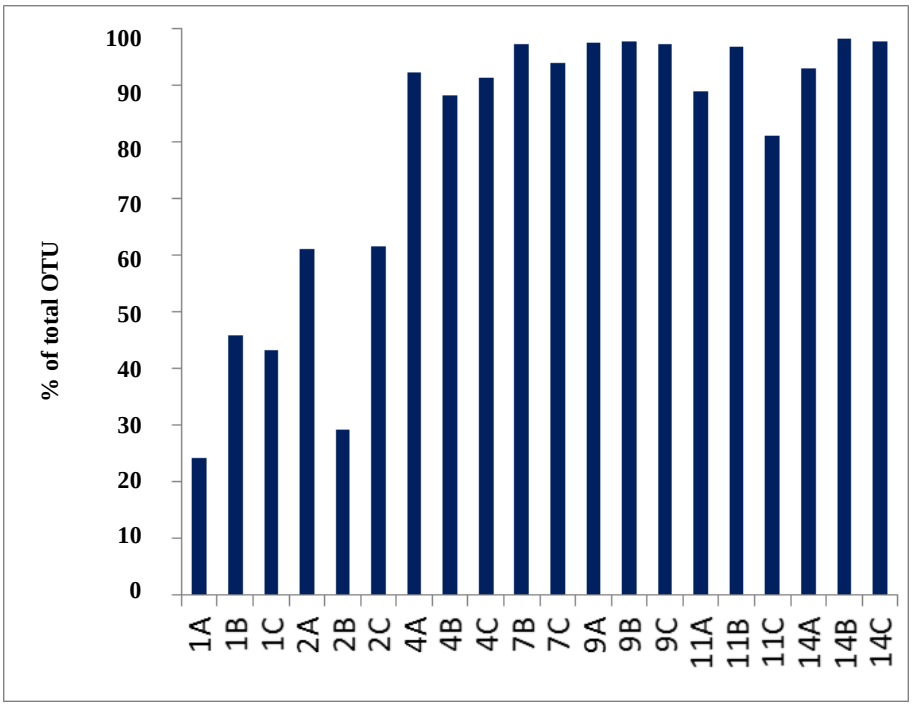

Fig. 3. Bacterial population (as %) that belongs to OUTs present in all samples during spoilage. In X-axis, the number represents spoilage time (in days) and letter the sample alanyzed.

The other major group of bacteria along the spoilage was *Pseudomonas*. Our results indicate that this group represented between 12 and 23% of the total bacterial population (Table 4), mainly corresponding to only one OTU (over 90% of all detected *Pseudomonas* are OTU0003). Some species of the genus *Pseudomonas* has been described before as important genus in lettuce phyllosphera (Rastogi et al., 2012; Jackson et al., 2013) and dominant in RTE lettuce (Rudi et al., 2002) and it may represent up to 90% of the microbial population in vegetables (Zagory, 1999). Our approach is not able to differentiate between *Pseudomonas*, though *P. fluorescens* was identified as mayor representative of this group in oak leaf lettuce (Nübling et al., 2016). The proportion of *Pseudomonas* was stable between 4 and 14 days of storage (Table 4), in between 16-24% of total bacterial load. Similar stability was observed by Ioannidis et al. (2018) in MAP Iceberg lettuce, where *Pseudomonas* reached 25-35% of the total population after 10 days.

Enterobacteriaceae (as "enteric_bacteria_cluster") increased their presence in samples up to 3.4±1.5 % at day 9. Compared with other published data, seems that this bacterial group is less important in Batavia lettuce compared with other varieties. Since, at harvesting time, *Enterobacteriaceae* was deemed the major group in Romaine lettuce (>38% of total bacteria) (Leff & Fierer, 2013), in lettuce produced both in spring and summer (Yu et al., 2018) and in

Iceberg lettuce (Ioannidis et al., 2018). The *Enterobacteriaceae* group includes many different organisms and was not defined up to the genus level in the informatics analysis. In the bibliography, most studies point to the important role of *Erwinia*, *Pantoea*, *Enterobacter*, and *Rahnella* in vegetables (Rudi et al., 2002; Rastogi et al., 2012). Conversely, we had identified *Erwinia* and *Pantoea* in our samples but at very low proportion (less than 0.1% and less than 0.5 % of the population, respectively) while we have not identified Rahnella, despite it have been underlined by Ioannidis et al. (2018) as one of the main bacteria in spoiled iceberg lettuce (up to 15-20% of total bacteria).

Finally, LAB (usually considered as *Lactobacillales*) were present in all samples during the storage, representing usually below 3% of total bacteria, in agreement with the ratio between counts in selective medium (MRS) and general medium (TVC). Main identified genera were *Lactococcus* and *Carnobacterium*, without statistical differences due to time of storage. In Iceberg lettuce, *Lactococcus* and *Leuconostoc* have been described as major LAB genus at spoilage time (Ioannidis et al., 2018). Leff and Feirer (2013) found *Leuconostaceae* as the major LAB family in Romaine lettuce, but we have found very low levels of this family in our samples and, surprisingly, *Lactobacillus* has not been identified in our samples.

Having in mind that OTUs were not identified at the species level, the presence of some pathogen bacteria in RTE lettuce at very low concentrations could not be excluded. In this regards, *Aeromonas* ssp has been identified at late spoilage time (up to 0.1% of the total population at day 14), *Yersinia* ssp represented up to 0.17%, some of the *Pseudomonas* ssp identified could be *P. aeroginosa* and the only OTU identified as *Bacillus ssp* would be a pathogenic species. All these species have been previously described as part of lettuce's bacteriome (Yu et al., 2018). We have not identified other usually pathogens, like *Escherichia*, *Salmonella*, *Listeria*, *Staphylococcus* or *Campilobacter*, probably due to the good sanitation effect of the post-harvest treatment.

The predominant specific spoilage microorganism (SSO) in RTE lettuce would be mainly *Pseudomonas*, *Enterobacteriacea*e and lactic acid bacteria (LAB). Although the proportion of these groups is highly variable, a similar situation can be observed in similar studies (i.e. Rudi et al., 2002). Strains of *Pseudomonas* and *Enteroacteriacieae* have been described as high-spoilers bacteria in RTE vegetables (Ragaert et al., 2006; Kahala et al., 2012; Federico et al., 2015) and, according to Ragaert et al. (2006), *Rahnella* and *Pantoea* (both *Enterobacteriaceae*) are able to produce spoilage-related volatile compounds in lettuce-agar. In the same direction, LAB are described as microorganisms with high spoilage capacity in these products (Pothakos et al., 2014). Within this group, *Leuconostoc* or *Lactobacillus* had been identified as some of the main causes of deterioration in salads (Nguyen-the & Carlin, 1994; Pothakos et al., 2014), but the levels reported hereby are below the, in theory, acceptable initial level (10^4 cfu.g^{-1}) (Jacxsens et al., 2003). *Flavobacterium*, the mayor genus, is described as no-SSO in fish (Parlapani et al., 2013), but some strains can produce aromas related with chicken spoilage (Freeman et al., 1976) and it has been proposed as spoilage bacteria in frozen vegetables (Manani et al., 2006). Further research is required to clarify the role of *Flavobacterium* in the spoilage of RTE salads. In general, detected relevant SSO microorganisms can grow at low temperatures and oxygen concentration, they are able to resist up to 10% CO_2 and are resistant to mild thermal treatments, which hinders the development of strategies to increase the shelf-life of RTE.

Hereby presented results showed a good correlation between the conclusions obtained by culture-dependent and independent methods. Bacterial counts in *Pseuodomonas* agar were in the same magnitude order than counts in TVC, and allowed us to identify *Pseudomonas* as one of the dominant genus identified in the population. Counts in selective agars for LAB (MRS) and *Enterobacteriaceae* (VRBG) and comparison with counts in TVC agree with

metagenomic results, concluding that these genera represent below 5% of total bacteria along the spoilage time. Jackson et al. (2013) also concluded that culture-dependent methods are a good overview of the bacterial populations present in RTE vegetables, while the advantage of culture-independent methods is that these techniques allow evaluating the importance of certain genera (like *Flavobacterium)* without a selective medium.

CONCLUSIONS

Sensorial analysis results indicate a commercial shelf-life of 9 days for Batavia lettuce stored in MAP at 6 ºC, mainly due to the apparition of off-odour after this period. The sensory limit of shelf life overlaps with bacterial counts in TVC and Pseudomonas agar reaching levels higher than 8 Log cfu.g^{-1}. Metagenomic analysis showed that *Flavobacteriales* (mainly *Flavobacterium* and *Chryseobacterium*) is the most abundant bacteria in RTE Batavia lettuce along with storage. *Pseudomonaceae* (especially *Pseudomonas*) is the second family in importance, and *Enterobacteriaceae* and LAB did not represent more than 5% of total obtained sequences. Finally, the microbial population is dominated by a few specific OTUs that were present throughout spoilage. These few OTUs may represent up to 98% of the total bacterial load.

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Conflict of interest
The authors declare no conflict of interest to report.

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