



Model development for microbial spoilage of packaged fresh-cut salad products using temperature and in-package CO₂ levels as predictor variables

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ABSTRACT

The objective of this study was to develop a model system for the prediction of the impact of temperature and in-package % CO₂ on the microbial spoilage of fresh-cut vegetables with high respiration rate independent of packaging characteristics and post-harvest physiology. Model development was based on study of rocket pulp stored under 0–20% O₂: 20–0% CO₂: 80% N₂ at 0–15 °C. Growth of pseudomonads and lactic acid bacteria (LAB) was primary modelled by Baranyi model, while growth rate was further modeled as a function of storage temperature and % CO₂ by a polynomial model. Both growth models were validated against various fresh-cut vegetables of high respiration rate, modified atmosphere packaging, and packaging films, under isothermal and dynamic temperature conditions. Variations of gas concentration of rocket pulp was eliminated or significantly decreased ($p < 0.05$), verifying our initial hypothesis. Pseudomonads were the dominant spoilage microorganisms in rocket pulp, while LAB were significantly favored ($p < 0.05$), as temperature and % CO₂ increased. Both models had acceptable performance, presenting pRE (proportion of relative error) ≥ 0.70 , bias factor of 0.99–1.00 and accuracy factor of 1.04–1.07. The developed empirical models in rocket pulp may be a useful tool to predict pseudomonads and LAB behavior in commercial packages of different fresh-cut salads such as rocket, romaine, and iceberg lettuce.

1. Introduction

Over the last years, fresh-cut vegetables have gained worldwide popularity due to consumers demand for a healthier lifestyle by including fresh foods in their daily nutrition. Given the post-harvest in-package respiratory activity of fresh produce, their microbial spoilage and quality degradation is a dynamic multiparameter process (Del Nobile, Baiano, Benedetto, & Massignan, 2006; Lee, Arul, Lencki, & Castaigne, 2010). In case of microbial spoilage, a critical population (i.e., 8.0–9.0 log CFU/g) of the dominant microorganism (Specific Spoilage Organism) will determine the end of microbial shelf-life of fresh-cut salads. From technological aspect, modified atmosphere packaging (MAP) is commonly used to extend the shelf-life of such perishable food products (Sandhya, 2010). MAP may also control the respiration rate of packaged vegetables by establishing an in-package gas atmosphere of reduced O₂ and increased CO₂ levels, surrounding the product, thus retarding product senescence and deterioration, while also limiting microbial growth (Fonseca, Oliveira, & Brecht, 2002; Kader, Zagory, & Kerbel, 1989). According to previous literature data, the recommended concentrations of O₂ in MAP during storage of

fresh-cut vegetables should range between 1 and 5% (Sandhya, 2010), whereas CO₂ concentrations should exceed 10% (Zagory & Kader, 1988), in order to diminish respiration rate, decrease the deterioration rate and the occurrence of aerobic bacteria, and consequently extend the shelf-life of various fresh-cut vegetables (Babic & Watada, 1996; Ballantyne, Stark, & Selman, 1988; Brecht, 1980; Kader, 1986; Kader & Saltveit, 2002; Solomos & Kanellis, 1989; Zagory & Kader, 1988). The initial gas composition changes dynamically during storage until a gas equilibrium is established around the product, which composition must be as close as possible to the optimal one, so as to reduce respiration, prevent ripening, senescence fermentation (Fonseca et al., 2002; Gontard & Guillaume, 2009; Oliveira et al., 2015). This concept has been further developed into the so-called “equilibrium modified atmosphere” (Jacxsens, Devlieghere, De Rudder, & Debevere, 2000; Jacxsens, Devlieghere, & Debevere, 2002). The gaseous equilibrium is affected by various factors such as film characteristics (surface area; gas and water vapor permeability), storage temperature, free (i.e., not occupied by food) volume inside the package, weight and respiration rate of vegetable as well as the initial gaseous composition (Fonseca et al., 2002). Along with gas composition, control of storage temperature is also

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critical for minimizing the effect of rapid respiratory metabolism of fresh-cut vegetables (Zhu et al., 2018).

In order to assess quantitatively the effect of the aforementioned factors on microbial growth, the use of predictive models is proposed as a powerful decision-supporting tool for the fresh produce industry, capable of determining the remaining shelf-life of fresh-cut salads. Numerous studies have researched the microbial spoilage of fresh-cut vegetables such as baby spinach, iceberg lettuce, broccoli, carrots, soybean sprouts, wild rocket, yet, without further modeling the generated experimental data (Allende, Luo, McEvoy, Artés, & Wang, 2004; Ansah, Amadio, & Colelli, 2015; Hyun & Lee, 2018; Mudau, Soundy, Araya, & Mudau, 2018; Paillart et al., 2017). Even though predictive models, validated under commercial packaging conditions, are available for lettuce, carrots, or mixed salads (lettuce, carrot, and red cabbage; lollo rosso lettuce, lollo verde lettuce, and rocket), their applicability is constrained to the product and packaging conditions for which they were developed (García-Gimeno & Zurera-Cosano, 1997; Sinigaglia, Albenzio, & Corbo, 1999; Tsironi et al., 2017). In fact, only Tsironi et al. (2017) validated the developed growth models under dynamic temperature profiles, while none of the models developed by the aforementioned research groups, were tested for their applicability under different MAP conditions and vegetables.

Considering the above, the approach of the present study was first to develop a model system for packaged vegetables based on the collection of data describing exclusively the impact of temperature and CO₂ levels on microbial spoilage of fresh-cut salads, isolating other convoluted factors associated with packaging and post-harvest physiology of the plant tissue. Secondly, the models to be further used for predicting the microbial spoilage of a variety of fresh-cut salads in commercial packages, using as input only the fluctuations in temperatures and in-package CO₂ (flushed or produced) during storage, regardless of the respiration rate of packaged vegetables and packaging characteristics (e.g., film permeability, headspace, etc.). To achieve the above we sought: i) to evaluate the developed food – packaging model system via monitoring gases concentrations during storage at different temperatures and MAPs in comparison to rocket salad tested under the same set-up, ii) to fit empirical growth models for the most important microbial groups such as pseudomonads and lactic acid bacteria (LAB) as a function of temperature and % CO₂ on the selected food model (rocket pulp), and iii) to validate the developed growth models on various commercial bagged fresh-cut vegetables of high respiration rate (rocket, romaine lettuce, and iceberg lettuces), MAPs, packaging films (of low and high permeability) at different storage temperature conditions (isothermal and dynamic).

2. Materials and methods

2.1. Experimental design

Commercial packages of ready to eat (RTE) fresh-cut rocket salad (*Eruca sativa* L.) (80 g/ package) were provided directly by a leading Greek fresh-cut salad producing and packaging industry in order to exclude distribution chain and any potential temperature abuse. Rocket was chosen as the model food due to its high respiration rate ca. 58–135 mg CO₂/kg · h at 5 °C (Siomos & Koukounaras, 2007; Mahajan, Luca, & Edelenbos, 2016), thus being a good representative of a highly dynamic system of O₂/CO₂ fluctuations at post-harvest level, among other fresh-cut vegetables. As already mentioned, the equilibrium of gas concentration when a fresh-cut salad is packaged under modified atmosphere is a result of: a) respiration rate of the vegetable (parameter N°1), b) gas permeability of the packaging film (parameter N°2), and c) microbial growth rate (parameter N°3). A vegetable – packaging model system was set up to minimize the effect of the aforementioned parameters (Fig. 1) and enable data collection only for the impact of temperature and initially flushed CO₂ levels on microbial growth as follows: rocket salad was unpacked from its commercial package and

mechanical destruction of plant tissue was performed by blending rocket leaves under aseptic conditions (Waring Pro, HGB50E1, Torrington, CT, USA) until a homogeneous pulp was derived (this aimed at minimizing parameter N°1). Selection of low permeability packaging film aimed at minimizing parameter N°2. An increased headspace volume proportionally to sample size was used inside the package to deliberately delay any changes in gases derived by the microbial respiration (parameter N°3). Rocket pulp (10 g) was weighted and placed in packaging plastic bags (23 cm × 26 cm) with gas permeability ca. 25, 90, and 6 cm³/m² per day/10⁵ Pa for CO₂, O₂, and N₂ at 20 °C and 50% relative humidity (Flexo-Pack S.A., Athens, Greece). Five different MAP compositions were applied by using a gas flush, sealing packaging machine (Henko Vac 1900 Machine, Howden Food Equipment B.V., The Netherlands): i) 0% O₂; 20% CO₂; 80% N₂, ii) 5% O₂; 15% CO₂; 80% N₂, iii) 10% O₂; 10% CO₂; 80% N₂, iv) 15% O₂; 5% CO₂; 80% N₂, and v) 20% O₂; 0% CO₂; 80% N₂. Although gas concentrations of 1–5% O₂ and > 10% CO₂ have been recommended for extending the shelf-life of fresh-cut salads (Sandhya, 2010; Zagory & Kader, 1988), the broader range of the aforementioned gas compositions was selected for modelling purposes. All samples were stored at controlled isothermal conditions of 0, 5, 10, and 15 °C in high precision (± 0.5 °C) incubation chambers (MIR-153, Sanyo Electric Co., Osaka, Japan). Two independent storage experiments were performed and duplicate samples were used for each trial (n = 4). Each sample derived from a different package and two independent packages were analyzed at each sampling point. Samplings were performed every 4–5 days at 0 °C, 3–4 days at 5 °C, 2–3 days at 10 °C, and every 1–2 days at 15 °C. The same set up was run for 10 g of intact rocket salad as for rocket pulp packaged under the same conditions monitoring gases variation inside the packaging and microbial growth to confirm our hypothesis regarding the reduction of respiration rate by disrupting plant tissue and to verify the expected different microbial behavior between rocket pulp and rocket salad, respectively. Specifically, the microbial growth in rocket pulp was faster than in rocket salad, regardless of microbial group, temperature and MAP, since moisture and nutrients released from the pulp are likely more supportive to microbial growth, compared to the less available nutrients and the lower moisture content of the intact vegetable surface (data not shown).

2.2. Evaluating the developed food – packaging model system of rocket pulp via gases measurement

Concentrations (% v/v) of O₂ and CO₂ in the package headspace of rocket pulp and rocket salad were monitored using a portable PBI Dansensor A/S (Check Mate 9900 O₂/CO₂; Ringsted, Denmark) analyzer (accuracy ± 0.1%) by sampling 3 mL of gas from the package headspace with a medical type needle. The needle was inserted in the headspace via a septum glued on the external part of the MAP pouch. Septum was used to prevent crack propagation in the film package due to the puncture (Charles, Guillaume, & Gontard, 2008; Guillaume, Guehi, Gontard, & Gastaldi, 2013).

2.3. Microbiological analysis

Ten (10) g of rocket pulp and 90 mL of sterile ¼ strength Ringer's solution (Lab M, Lancashire, UK) were homogenized in a stomacher (Interscience, France) for 60 s. Following homogenization, decimal dilutions in ¼ strength Ringer's solution were prepared and 1 or 0.1 mL of the appropriate dilution was poured or spread, respectively, on selective and non-selective culture media. Total Viable Counts (TVC) were enumerated on Plate Count Agar (PCA; Lab M, Lancashire, UK) after incubation at 30 °C for 48 h; Lactic Acid Bacteria (LAB) were determined on double layer of De Man, Rogosa and Sharp agar (MRS; Lab M Limited, Bury, UK) (pH 5.8) incubated at 30 °C for 72 h; *Pseudomonas* spp. were enumerated on *Pseudomonas* Selective Agar (Lab M, Lancashire, UK) with Cetrimide – Fucidin – Cephaloridine supplement

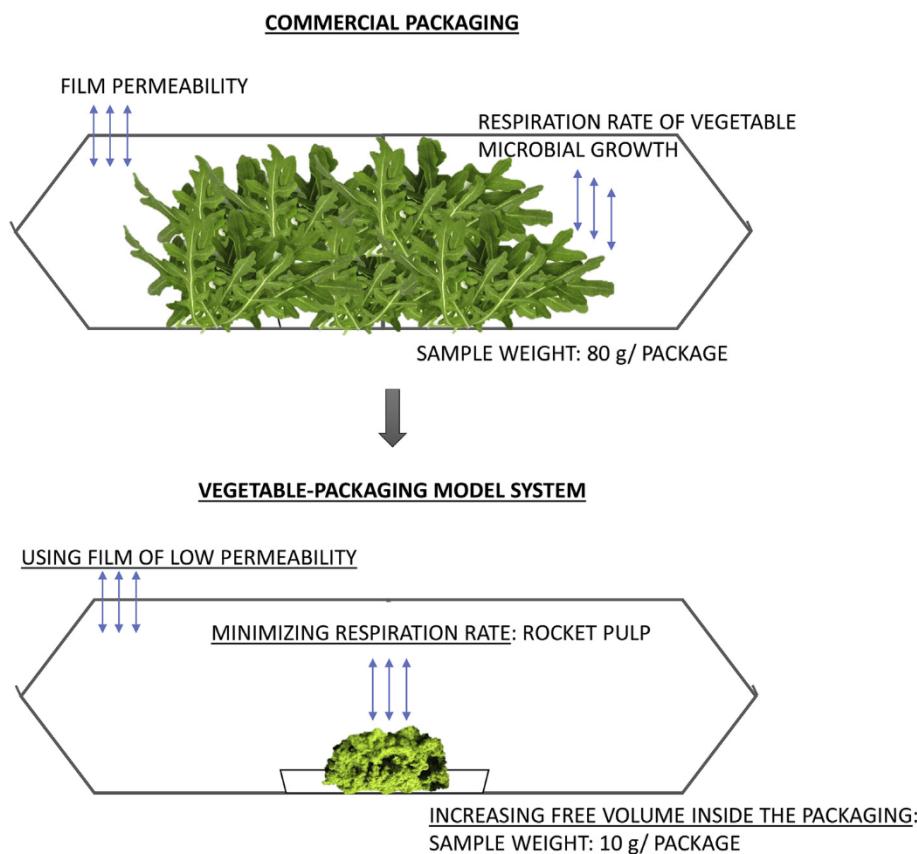


Fig. 1. Schematic representation of the developed food – packaging model system for empirically predicting spoilage of fresh-cut vegetable salads under different CO_2 concentrations and storage temperatures *vice versa* the commercial packaging of rocket salad.

Table 1

Validation experiments in rocket, romaine lettuce, and iceberg lettuce performed in order to test the efficacy of the developed empirical model.

Validation experiment	Food substrate	Packaging film	Modified atmosphere packaging	Storage temperature
N°1	Rocket (10 g/package)	Experimental ^a	0% O_2 : 20% CO_2 ; 80% N_2 10% O_2 : 10% CO_2 ; 80% N_2 20% O_2 : 0% CO_2 ; 80% N_2	Dynamic (8 h at 8 °C, 8 h at 12 °C, and 8 h at 4 °C)
N°2	Rocket (110 g/package)	Commercial ^b	3% O_2 : 10% CO_2 ; 87% N_2	Isothermal: 5 °C and 12 °C
N°3	Rocket (80 g/package)	Commercial ^c	3% O_2 : 10% CO_2 ; 87% N_2	Dynamic ($T_{\text{eff}} = 4.2$ °C and $T_{\text{eff}} = 6$ °C)
N°4	Romaine lettuce (200 g/package)	Commercial ^c	3% O_2 : 10% CO_2 ; 87% N_2	Dynamic (8 h at 8 °C, 8 h at 12 °C, and 8 h at 4 °C)
N°5	Iceberg lettuce (200 g/package)	Commercial ^c	3% O_2 : 10% CO_2 ; 87% N_2	Isothermal: 5 °C

^a Plastic bags (23 cm × 26 cm; gas permeability *ca.* 25, 90, and 6 cm^3/m^2 per day/ 10^5 Pa for CO_2 , O_2 , and N_2 at 20 °C and 50% relative humidity).

^b Unknown.

^c Laser micro-perforated commercial packages (oriented polypropylene OPP COEX perforated ANTIFOG film bags with 30 µm thickness and an O_2 permeability of 9000 cm^3/m^2 24 h atm).

(CFC; Lab M, Lancashire, UK) agar after incubation at 25 °C for 72 h; yeasts and molds were determined on Rose Bengal Chloramphenicol Agar (RBC; Lab M, Lancashire, UK) incubated at 25 °C for 4–5 days; Enterobacteriaceae were counted on a double layer of Violet Red Bile Glucose Agar (VRBGA; Lab M, Lancashire, UK) incubated at 37 °C for 24 h. The pH values of rocket pulp samples were recorded after each microbiological sampling by using a digital pH meter (pH 526, Metrohm Ltd, Switzerland) immersed in the homogenate after microbiological analysis.

2.4. Primary modelling

Although the indigenous microbiota of fresh-cut vegetable salads consists of several microbial groups, *Pseudomonas* spp. and LAB were selected to be modeled due to their dominance and their recorded high growth potential, respectively. Growth curves were generated by

plotting bacterial population ($\log \text{CFU/g}$) vs storage time. Maximum specific growth rate (μ_{\max} ; days $^{-1}$), lag time (λ , days), initial microbial load N_0 ($\log \text{CFU/g}$) and maximum microbial load N_{\max} ($\log \text{CFU/g}$) per combination of % CO_2 and storage temperature were estimated using the Baranyi growth model *via* DMFit (available at <http://www.combase.cc/index.php/en/>) (Baranyi & Roberts, 1994).

2.5. Secondary modelling

The effect of storage temperature and % CO_2 on growth of *Pseudomonas* spp. and LAB in rocket pulp (model matrix) was modeled with a polynomial equation *via* DMFit. Natural logarithm transformations of μ_{\max} were selected among square root and no transformation for reducing the variance of the response variable, as generated by the Baranyi model, and provided the best fit (Eq. (1)). In the following equation, α_0 , α_1 , α_2 , α_3 , α_4 , and α_5 are the regression coefficients, while

Table 2

Estimated kinetic parameters (N_0 (log CFU/g); N_{\max} (log CFU/g); μ_{\max} (days $^{-1}$); λ (days)) of LAB, derived from fitting the Baranyi model in rocket pulp packaged under different MAP and stored at 0, 5, 10, and 15 °C.

MAP	Temperature (°C)	LAB			
		N_0 (log CFU/g) ± stdev	N_{\max} ± stdev (log CFU/g)	μ_{\max} ± stdev (days $^{-1}$)	λ ± stdev (days)
0% O ₂ : 20% CO ₂	0	2.84 ± 0.08 ^{Aa}	5.49 ± 0.50 ^{Ab}	0.21 ± 0.04 ^{Aa}	-
	5	2.70 ± 0.00 ^{Aa}	7.25 ± 0.08 ^{Bc}	0.58 ± 0.00 ^{Ba}	-
	10	2.73 ± 0.01 ^{Aa}	7.75 ± 0.11 ^{Bd}	0.99 ± 0.01 ^{Cbc}	-
	15	2.90 ± 0.30 ^{Aa}	7.68 ± 0.16 ^{Bc}	1.73 ± 0.19 ^{Da}	-
5% O ₂ : 15% CO ₂	0	3.00 ± 0.31 ^{Aa}	5.46 ± 0.04 ^{Ab}	0.19 ± 0.00 ^{Aa}	13.66 ± 2.44
	5	3.06 ± 0.01 ^{Ac}	6.39 ± 0.34 ^{B^b}	0.51 ± 0.06 ^{Ba}	-
	10	2.95 ± 0.02 ^{Aa}	7.39 ± 0.15 ^{Cc}	0.84 ± 0.01 ^{Ca}	-
	15	3.14 ± 0.01 ^{Aa}	7.81 ± 0.06 ^{Cc}	1.59 ± 0.10 ^{Da}	-
10% O ₂ : 10% CO ₂	0	2.77 ± 0.08 ^{Aa}	6.02 ± 0.24 ^{Abc}	0.22 ± 0.07 ^{Aa}	1.33 ± 0.02
	5	2.70 ± 0.00 ^{Aa}	6.48 ± 0.06 ^{ABb}	0.49 ± 0.01 ^{Ba}	-
	10	2.69 ± 0.00 ^{Aa}	6.89 ± 0.00 ^{BCb}	1.06 ± 0.01 ^{Cc}	-
	15	3.78 ± 0.04 ^{Aa}	7.06 ± 0.07 ^{Cb}	1.73 ± 0.01 ^{Da}	-
15% O ₂ : 5% CO ₂	0	2.87 ± 0.03 ^{Aa}	6.62 ± 0.00 ^{Ac}	0.19 ± 0.03 ^{Aa}	13.50 ± 0.07
	5	2.85 ± 0.07 ^{Ab}	7.50 ± 0.11 ^{Bc}	0.57 ± 0.02 ^{Ba}	-
	10	2.87 ± 0.00 ^{Ab}	7.89 ± 0.08 ^{Bd}	0.87 ± 0.02 ^{Cab}	-
	15	3.16 ± 0.41 ^{Aa}	7.62 ± 0.22 ^{Bc}	1.68 ± 0.03 ^{Da}	-
20% O ₂ : 0% CO ₂	0	2.70 ± 0.00 ^{Aa}	3.86 ± 0.24 ^{Aa}	0.19 ± 0.05 ^{Aa}	-
	5	2.70 ± 0.01 ^{Aa}	5.67 ± 0.00 ^{Ba}	0.47 ± 0.06 ^{Ba}	-
	10	2.69 ± 0.02 ^{Aa}	5.93 ± 0.02 ^{BCa}	1.03 ± 0.08 ^{Cc}	-
	15	2.74 ± 0.04 ^{Aa}	6.28 ± 0.03 ^{Ca}	1.62 ± 0.11 ^{Da}	-

1 N_0 , N_{\max} and μ_{\max} values within the same MAP having different uppercase letter are significantly different from each other ($p < 0.05$).

2 N_0 , N_{\max} and μ_{\max} values within the same temperature having different lowercase letter are significantly different from each other ($p < 0.05$).

Table 3

Estimated kinetic parameters (N_0 (log CFU/g); N_{\max} (log CFU/g); μ_{\max} (days $^{-1}$); λ (days)) of pseudomonads, derived from fitting the Baranyi model in rocket pulp packaged under different MAP and stored at 0, 5, 10, and 15 °C.

MAP	<i>Pseudomonas</i> spp.				
	Temperature (°C)	N_0 (log CFU/g) ± stdev	N_{\max} ± stdev (log CFU/g)	μ_{\max} ± stdev (days $^{-1}$)	λ ± stdev (days)
0% O ₂ : 20% CO ₂	0	6.23 ± 0.09 ^{Ab}	N.G. ^{Aa}	N.G. ^{Aa}	N.G.
	5	6.40 ± 0.09 ^{ABbc}	N.G. ^{Aa}	N.G. ^{Aa}	N.G.
	10	6.74 ± 0.22 ^{ABb}	N.G. ^{Aa}	N.G. ^{Aa}	N.G.
	15	6.94 ± 0.08 ^{Bc}	N.G. ^{Aa}	N.G. ^{Aa}	N.G.
5% O ₂ : 15% CO ₂	0	5.89 ± 0.04 ^{Aa}	7.59 ± 0.00 ^{Ab}	0.12 ± 0.03 ^{Aa}	10.82 ± 1.84
	5	6.18 ± 0.11 ^{ABab}	7.99 ± 0.21 ^{Ab}	0.18 ± 0.05 ^{ABab}	-
	10	6.20 ± 0.08 ^{Bab}	7.75 ± 0.12 ^{Ab}	0.32 ± 0.04 ^{Bb}	-
	15	6.17 ± 0.00 ^{ABA}	7.70 ± 0.00 ^{Ab}	0.53 ± 0.06 ^{Cb}	-
10% O ₂ : 10% CO ₂	0	6.62 ± 0.04 ^{Ac}	8.09 ± 0.28 ^{Abc}	0.12 ± 0.01 ^{Aa}	5.46 ± 1.12
	5	6.50 ± 0.05 ^{Ac}	8.22 ± 0.11 ^{Ab}	0.17 ± 0.03 ^{Aab}	-
	10	6.68 ± 0.19 ^{Aab}	8.66 ± 0.54 ^{Abc}	0.27 ± 0.03 ^{Bb}	-
	15	6.66 ± 0.07 ^{Abc}	8.14 ± 0.05 ^{Ac}	0.42 ± 0.04 ^{Cb}	-
15% O ₂ : 5% CO ₂	0	5.80 ± 0.05 ^{Aa}	7.92 ± 0.01 ^{Abc}	0.10 ± 0.03 ^{Aa}	6.59 ± 0.00
	5	6.00 ± 0.00 ^{ABA}	8.19 ± 0.16 ^{Ab}	0.19 ± 0.10 ^{Aab}	-
	10	6.12 ± 0.08 ^{ABA}	8.23 ± 0.21 ^{Ab}	0.49 ± 0.10 ^{Bc}	-
	15	6.13 ± 0.08 ^{Ba}	8.96 ± 0.15 ^{Bd}	1.14 ± 0.02 ^{Cc}	-
20% O ₂ : 0% CO ₂	0	6.85 ± 0.09 ^{Ac}	8.45 ± 0.24 ^{Ac}	0.10 ± 0.02 ^{Aa}	-
	5	6.72 ± 0.02 ^{Ad}	8.35 ± 0.10 ^{Ab}	0.42 ± 0.16 ^{ABb}	-
	10	6.54 ± 0.03 ^{Aab}	9.41 ± 0.03 ^{Bc}	0.56 ± 0.03 ^{Bc}	-
	15	6.59 ± 0.12 ^{Ab}	9.28 ± 0.03 ^{Be}	1.19 ± 0.14 ^{Cc}	-

N.G.: No Growth.

1 N_0 , N_{\max} and μ_{\max} values within the same MAP having different uppercase letter are significantly different from each other ($p < 0.05$).

2 N_0 , N_{\max} and μ_{\max} values within the same temperature having different lowercase letter are significantly different from each other ($p < 0.05$).

CO₂ (%) and T (°C) are the independent variables.

$$\ln(\mu_{\max}) = \alpha_0 + \alpha_1 * \text{CO}_2 + \alpha_2 * \text{T} + \alpha_3 * \text{CO}_2^2 + \alpha_4 * \text{T}^2 + \alpha_5 * \text{CO}_2 * \text{T} \quad (1)$$

2.6. Simulation and validation of the developed growth models of rocket pulp

Given that variable factors may occur in commercial packaged fresh-cut salads and some of them were isolated in the present study when the growth models were developed (see § 2.1), the main target was to perform several multi-parameter validation experiments instead

of biological replicates per validation experiment ($n = 2$) in order to test the response of the developed growth models to these parameters, each in isolation. Specifically, five validation experiments were designed in order to adequately validate the applicability of the developed growth models of LAB and *Pseudomonas* spp. against variable factors that may occur in commercial packaged fresh-cut salads. These factors include the weight of vegetable inside the packaging, initial gas concentrations in MAPs, storage temperature conditions (isothermal and dynamic) simulating potential temperature fluctuations during the supply chain, type of commercial packaging films (low and high permeability), as well as different fresh-cut vegetable salads of high

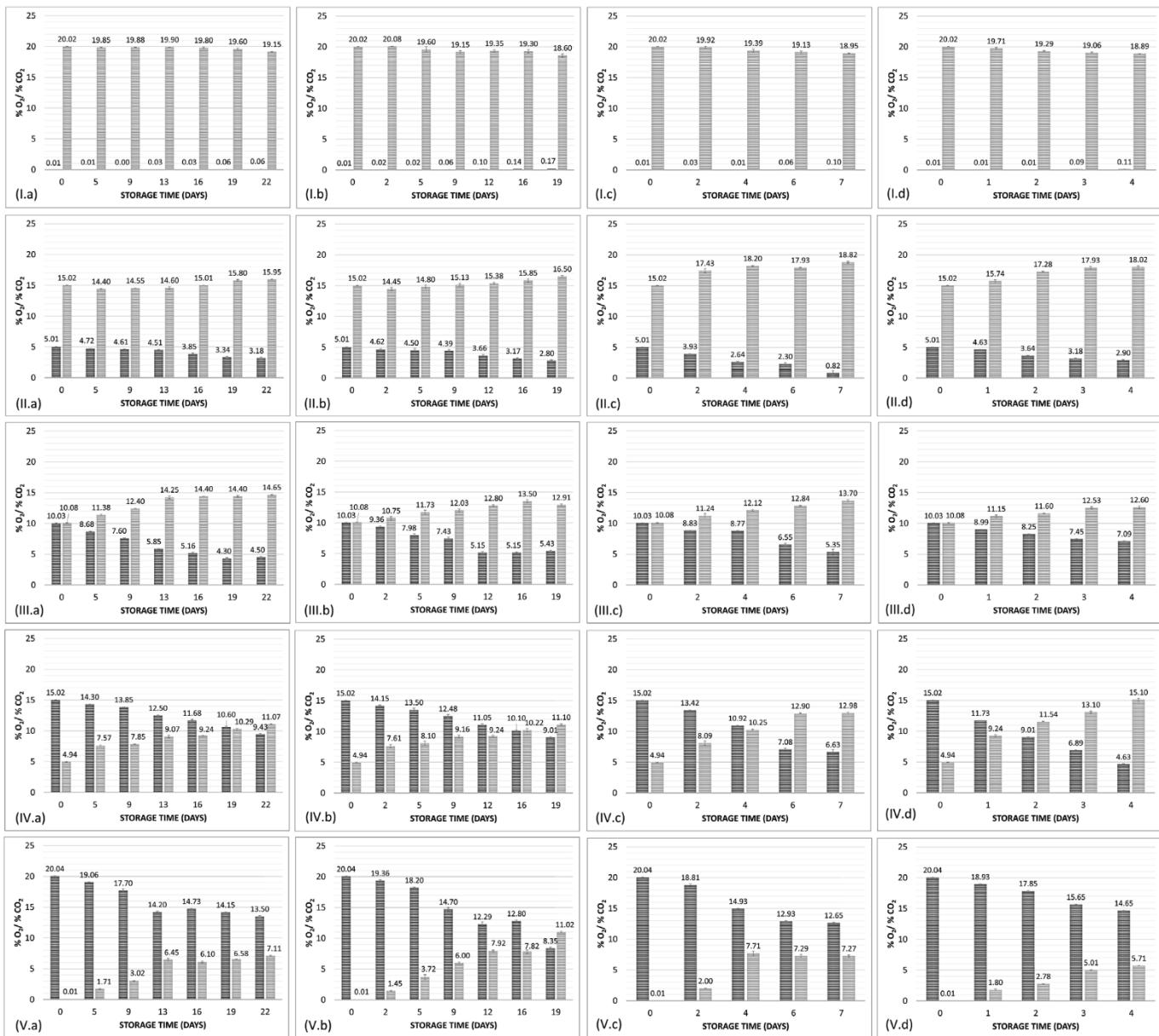


Fig. 2. Evolution of gas composition (% O₂: dark grey bar and % CO₂: light grey bar) in rocket salad packaged under (I) 0% O₂; 20% CO₂; 80% N₂, (II) 5% O₂; 15% CO₂; 80% N₂, (III) 10% O₂; 10% CO₂; 80% N₂, (IV) 15% O₂; 5% CO₂; 80% N₂, and (V) 20% O₂; 0% CO₂; 80% N₂ during isothermal storage at (a) 0 °C, (b) 5 °C, (c) 10 °C, and (d) 15 °C.

respiration rate (rocket, romaine lettuce, and iceberg lettuce). The experimental conditions under which the different validation studies were carried out are analytically presented in Table 1. The storage of fresh-cut salads packages took place in high precision (± 0.5 °C) incubation chambers (MIR-153, Sanyo Electric Co., Osaka, Japan). Temperature fluctuations were recorded by a computer downloadable electronic data logger (testo 174, Testo Inc.) placed inside the incubators, while percentage of CO₂, and microbiological parameters (pseudomonads and LAB) were monitored as described in § 2.2 and § 2.3.

The prediction of pseudomonads and LAB growth was based on the time-temperature profile obtained by the data loggers placed inside the incubators and the in-package CO₂ percentage was monitored as described in § 2.2, in conjunction with the polynomial model (Eq. (1)) for the estimation of the “momentary” μ_{max} between two consecutive reads. The derived μ_{max} was then introduced into the Baranyi primary model for certain N₀, N_{max} and h₀, to simulate the growth under non-

isothermal and fluctuating in-package CO₂ levels. As the value of N_{max} was set the maximum determined population (log CFU/g) per experimental condition based on fitted data from isothermal experiments that were used for the above secondary model fitting. The initial concentration (N₀; log CFU/g) of pseudomonads and LAB, was used as a starting point of simulation as determined by the plate count method (§ 2.3). H₀ is a parameter characterizing the “adaptation work” needed by the cells to enter the exponential phase or else “relative lag” and is the product of μ_{max} and lag (Manios, Skiadaresis, Karavasilis, Drosinos, & Skandamis, 2009; Robinson, Ocio, Kaloti, & Mackey, 1998). For the simulation, h₀ was inserted with a low value, namely 0.1, since no lag was determined in most of the studied combinations of temperature and % CO₂ for both microorganisms. In fact, according to Tables 2 and 3, lag phase was determined only during storage at 0 °C, a temperature which was not taken into account during validation experiments.

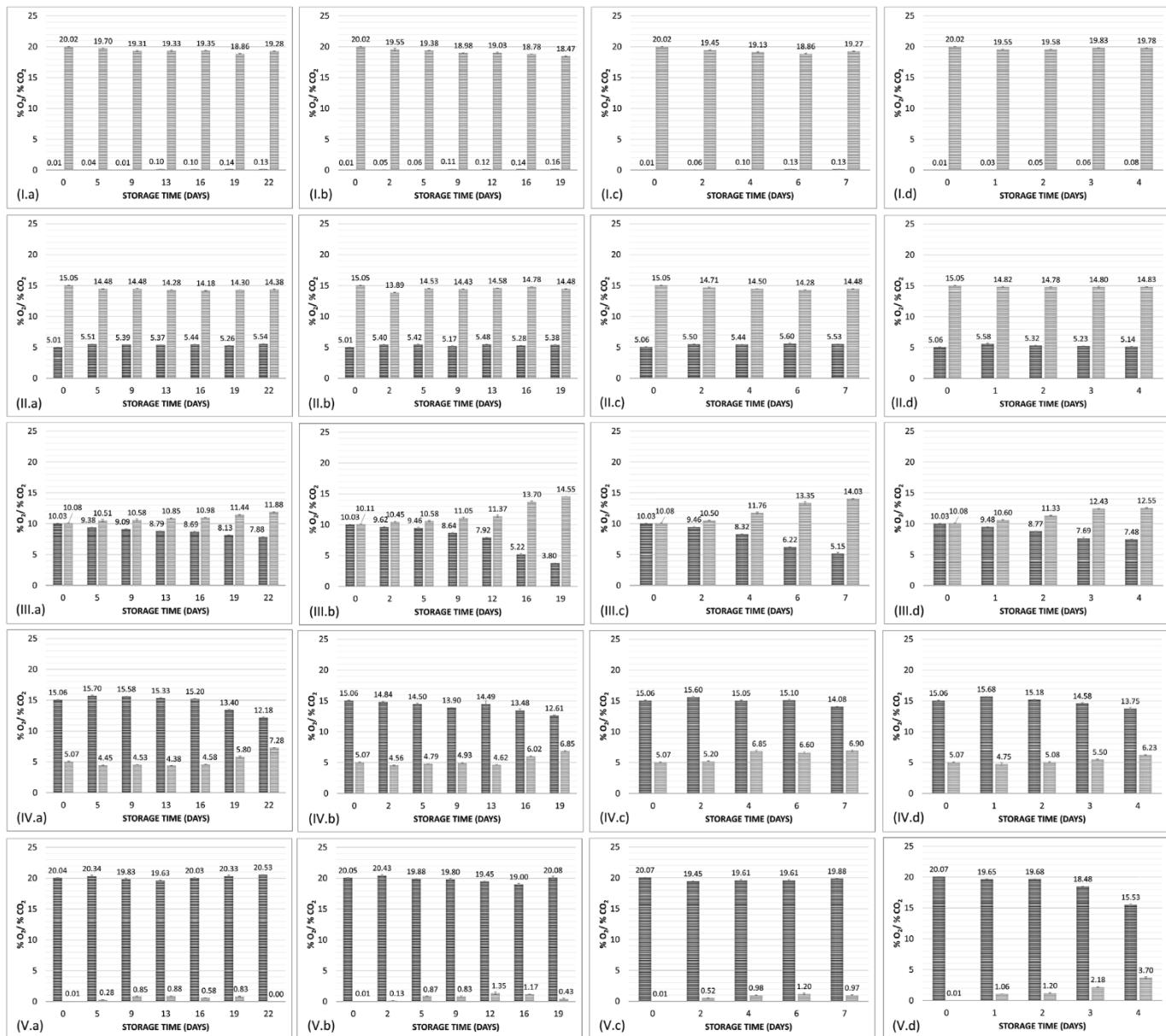


Fig. 3. Evolution of gas composition (% O₂: dark grey bar and % CO₂: light grey bar) in rocket pulp packaged under (I) 0% O₂; 20% CO₂; 80% N₂, (II) 5% O₂; 15% CO₂; 80% N₂, (III) 10% O₂; 10% CO₂; 80% N₂, (IV) 15% O₂; 5% CO₂; 80% N₂, and (V) 20% O₂; 0% CO₂; 80% N₂ during isothermal storage at (a) 0 °C, (b) 5 °C, (c) 10 °C, and (d) 15 °C.

2.7. Performance of the developed growth models of rocket pulp

To evaluate the performance of the models, the following statistical indicators were used: (i) adjusted regression coefficient (R_{adj}^2) and (ii) the root mean square error of the residuals of the model (RMSE). The higher the R_{adj}^2 value and the lower the RMSE value, the better was the goodness-of-fit of model.

The agreement between predicted and observed populations of pseudomonads and LAB was assessed by using the accuracy (Af) (Eq. (2)) and the bias (Bf) factors (Eq. (3)) (Ross, 1996). Specifically, Af and Bf were calculated separately by either taking into account the data per microorganism, % CO₂ condition, and validation experiment (Figs. 5–7) or by considering all data populations of pseudomonads and LAB regardless of % CO₂ condition and validation experiment (Fig. 8).

$$Af = 10^{\left[\frac{\sum_1^n \left| \log\left(\frac{\text{log } N_{\text{fitted}}}{\text{log } N_{\text{observed}}} \right) \right|}{n} \right]} \quad (\text{Eq. 2})$$

$$Bf = 10^{\left[\frac{\sum_1^n \log\left(\frac{\text{log } N_{\text{fitted}}}{\text{log } N_{\text{observed}}} \right)}{n} \right]} \quad (\text{Eq. 3})$$

where n equals to the number of observations. Perfect agreement between fitted and observed values is represented with accuracy and bias factors of 1 (Ross, 1996). Bf values of 1.00–1.15 indicate that predictions exceed observations (over-prediction), while values between 0.70 and 1.00 are interpreted as under-prediction (Ross, Dalgaard, & Tienungoon, 2000).

Likewise, relative error (RE) of LAB and pseudomonads populations (log CFU/g) of individual predictions were calculated (Delignette-Muller, Rosso, & Flandrois, 1995) by the following equation:

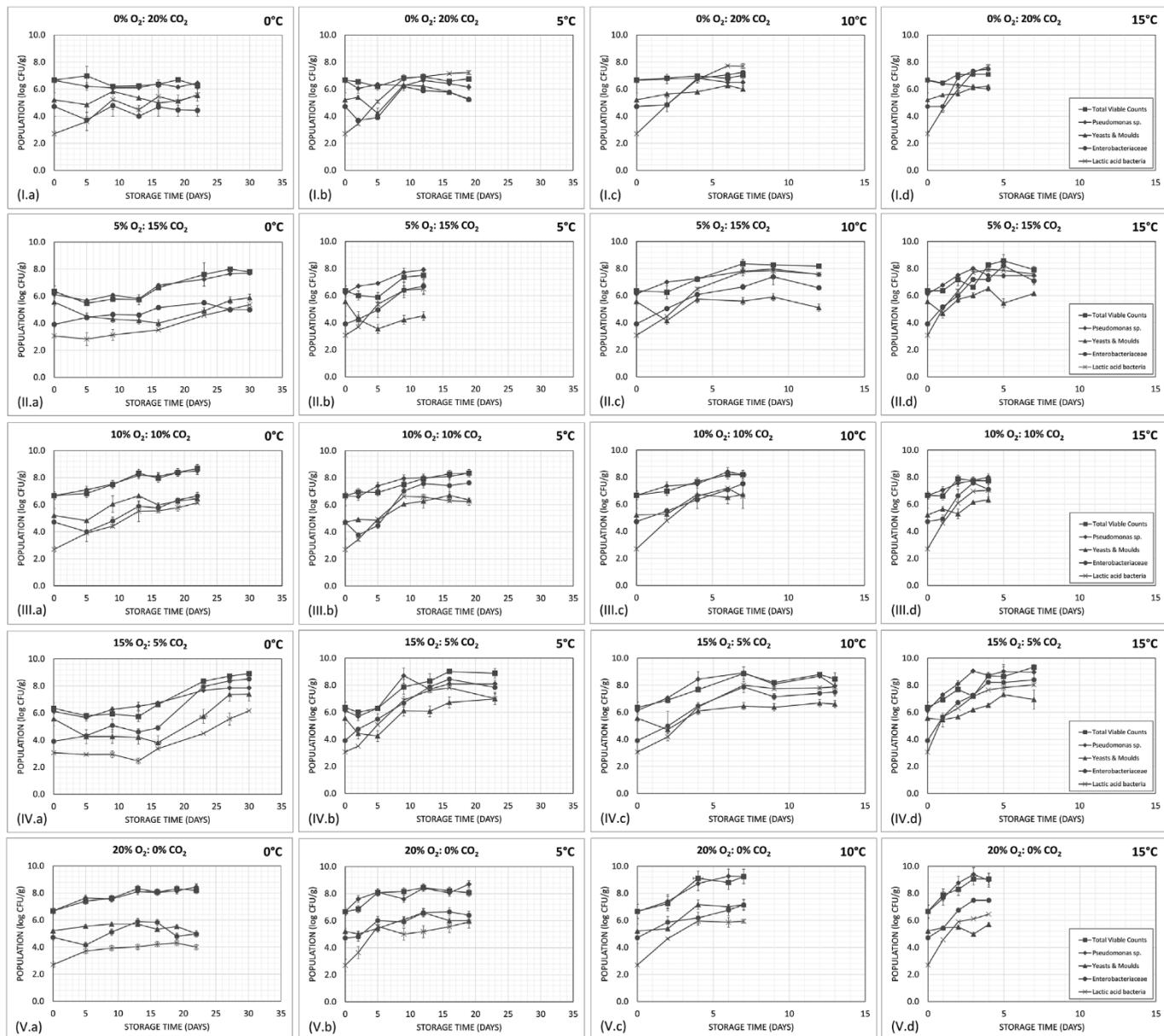


Fig. 4. Growth of LAB, pseudomonads, enterobacteria, yeasts and moulds, and TVC on rocket pulp packaged under (I) 0% O₂: 20% CO₂: 80% N₂, (II) 5% O₂: 15% CO₂: 80% N₂, (III) 10% O₂: 10% CO₂: 80% N₂, (IV) 15% O₂: 5% CO₂: 80% N₂, and (V) 20% O₂: 0% CO₂: 80% N₂ during isothermal storage at (a) 0 °C, (b) 5 °C, (c) 10 °C, and (d) 15 °C.

$$RE = (\text{observed} - \text{predicted}) / \text{predicted} \quad (4)$$

RE < 0 represented under-prediction and RE > 0 represented over-prediction of the developed models (Oscar, 2005). The proportion of RE (*pRE*) that fell in an acceptable prediction zone (namely the number of RE in the acceptable prediction zone/total number of prediction cases) from an RE of -0.3 (under-predictions) to 0.15 (over-predictions) was calculated and used also as a measure of model performance (Oscar, 2005). Models with *pRE* ≥ 0.70 were considered to provide predictions with acceptable bias and accuracy (Oscar, 2005).

2.8. Statistical analysis

Statistical analysis was performed with SPSS computer package Version 16.0 (SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was performed on data to evaluate the effect of MAP conditions and storage temperature on gases concentration and microbial

levels. Tukey's multiple range test was used for mean comparison. Statistical significance was assigned at *p* < 0.05.

3. Results and discussion

3.1. Evaluating the developed food – packaging model system of rocket pulp via gases measurement

Changes of gas composition in the headspace of rocket pulp and rocket salad packaged under 0–20% CO₂ and stored at 0, 5, 10, and 15 °C were monitored in order to study our hypothesis that by controlling critical parameters like respiration rate of vegetables, film permeability, and increased free volume inside the package, gas variation can be minimized during storage (Figs. 2 and 3). Comparing rocket pulp and rocket salad, the recorded pattern of gases variation is different. Gas concentration in the package significantly (*p* < 0.05) changed throughout storage of rocket salad, at almost all studied

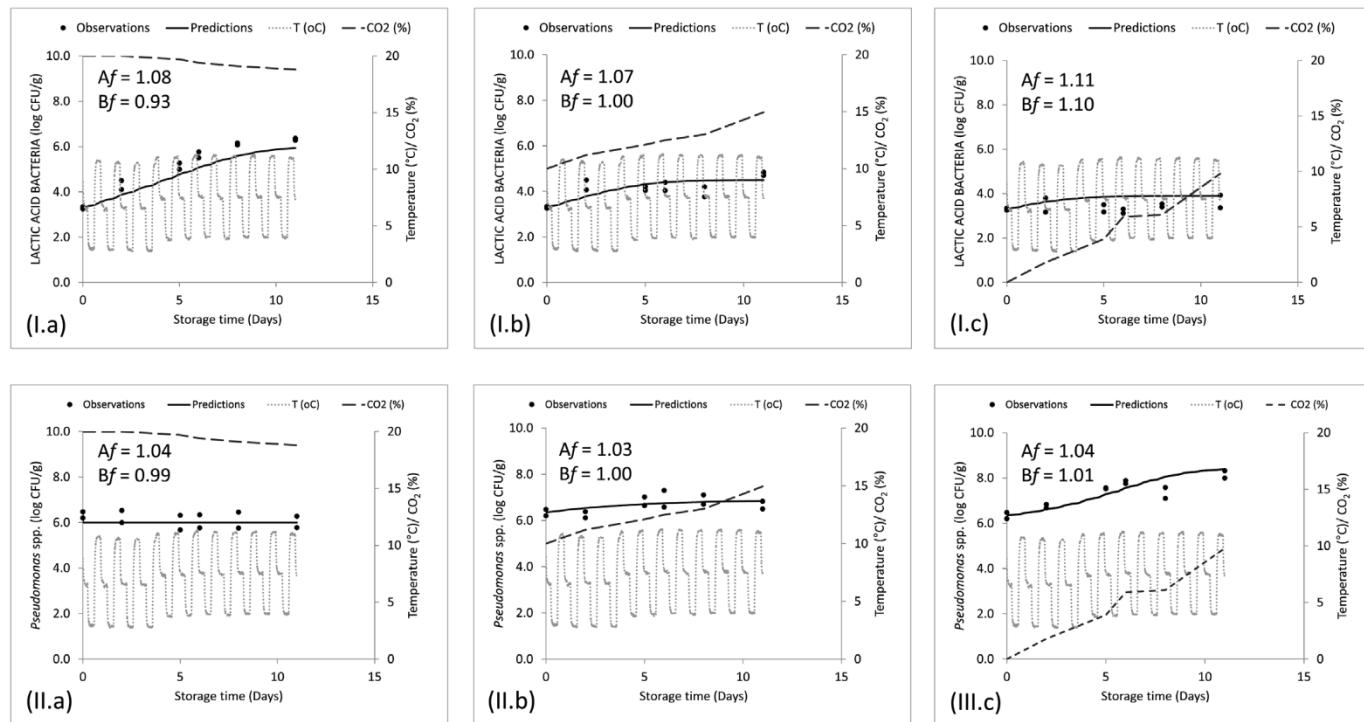


Fig. 5. Simulated growth curves and observed data (I) LAB and (II) pseudomonads in RTE fresh-cut rocket salad (10 g/package) packaged under (a) 0% O₂: 20% CO₂: 80% N₂, (b) 10% O₂: 10% CO₂: 80% N₂, and (c) 20% O₂: 0% CO₂: 80% N₂ in plastic bags (23 cm × 26 cm; gas permeability ca. 25, 90, and 6 cm³/m² per day/10⁵ Pa for CO₂, O₂, and N₂ at 20 °C and 50% relative humidity) and stored at dynamic temperature (8 h at 8 °C, 8 h at 12 °C and 8 h at 4 °C).

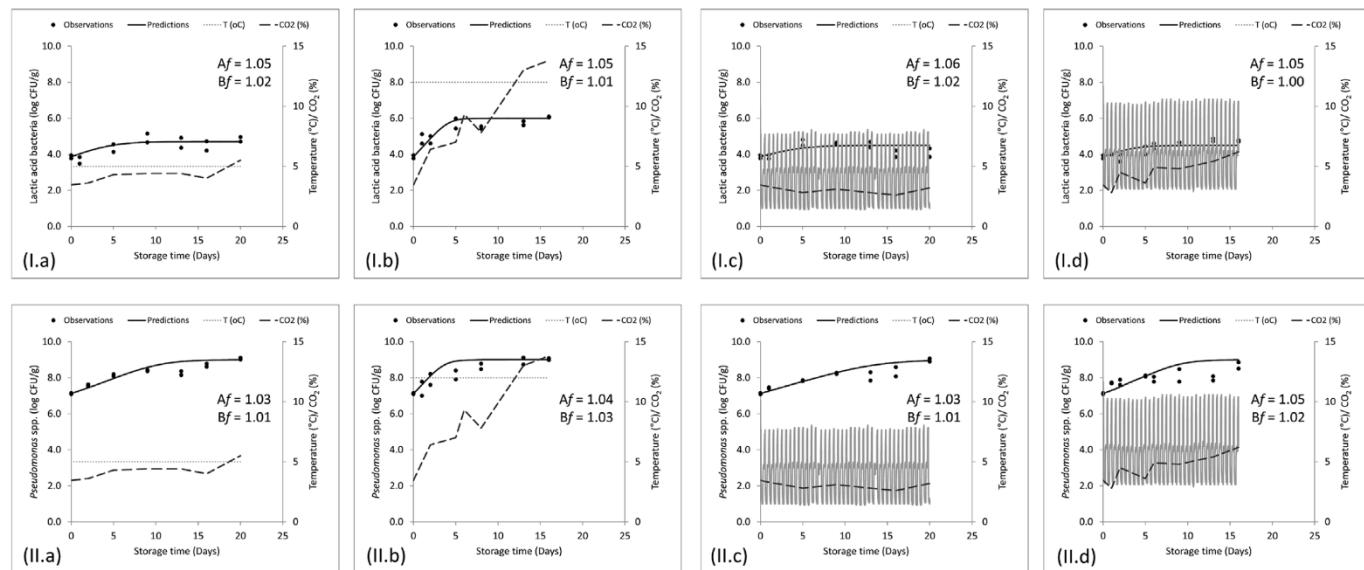


Fig. 6. Simulated growth curves and observed data of (I) LAB and (II) pseudomonads in commercially RTE fresh-cut rocket salad (110 g/package) packaged under 3% O₂: 10% CO₂: 87% N₂ in commercial packages stored at two isothermal [(a) 5 °C and (b) 12 °C] and two dynamic temperature profiles [(c) T_{eff} = 4.2 °C and (d) T_{eff} = 6 °C].

assays, as opposed to rocket pulp. The lowest variation was recorded under MAP of 0% O₂: 20% CO₂: 80% N₂ at all temperatures and 5% O₂: 15% CO₂: 80% N₂ during storage at 0 and 5 °C (Fig. 2(I.a) – (I.d) and 2(I.a) – (I.b)). It is well-known that the use of MAP with low O₂ percentage ($\leq 5\%$ O₂) causes reduction in respiration rate, inhibition of aerobic microorganisms, weight loss, and antioxidant capacity loss (Gil, Ferreres, & Tomás-Barberán, 1999; Izumi, Nonaka, & Muraoka, 1997; Ko, Watada, Schlimme, & Bouwknegt, 1996). Hence, 1–5% O₂ and $> 10\%$ CO₂ is recommended as the most effective gas concentrations during storage of commercial fresh-cut vegetables (Sandhya, 2010).

These attributes were clearly maintained on rocket pulp, namely no significant changes of the initial values of O₂ and CO₂ composition ($p \geq 0.05$) were recorded under the recommended aforementioned MAP conditions, regardless of storage temperature (Fig. 3(I.a) – (I.d) and (II.a) – (II.d)). The latter observation was obviously a combined result of the reduced respiration rate due to the low initial O₂ percentage ($\leq 5\%$ O₂) and the disruption of rocket leaves. On the contrary, rocket pulp packaged under MAP of 10% O₂: 10% CO₂: 80% N₂ showed significant decrease of % O₂ and increase of % CO₂ ($p < 0.05$) during storage, especially as storage temperature increased from 0 to 15 °C

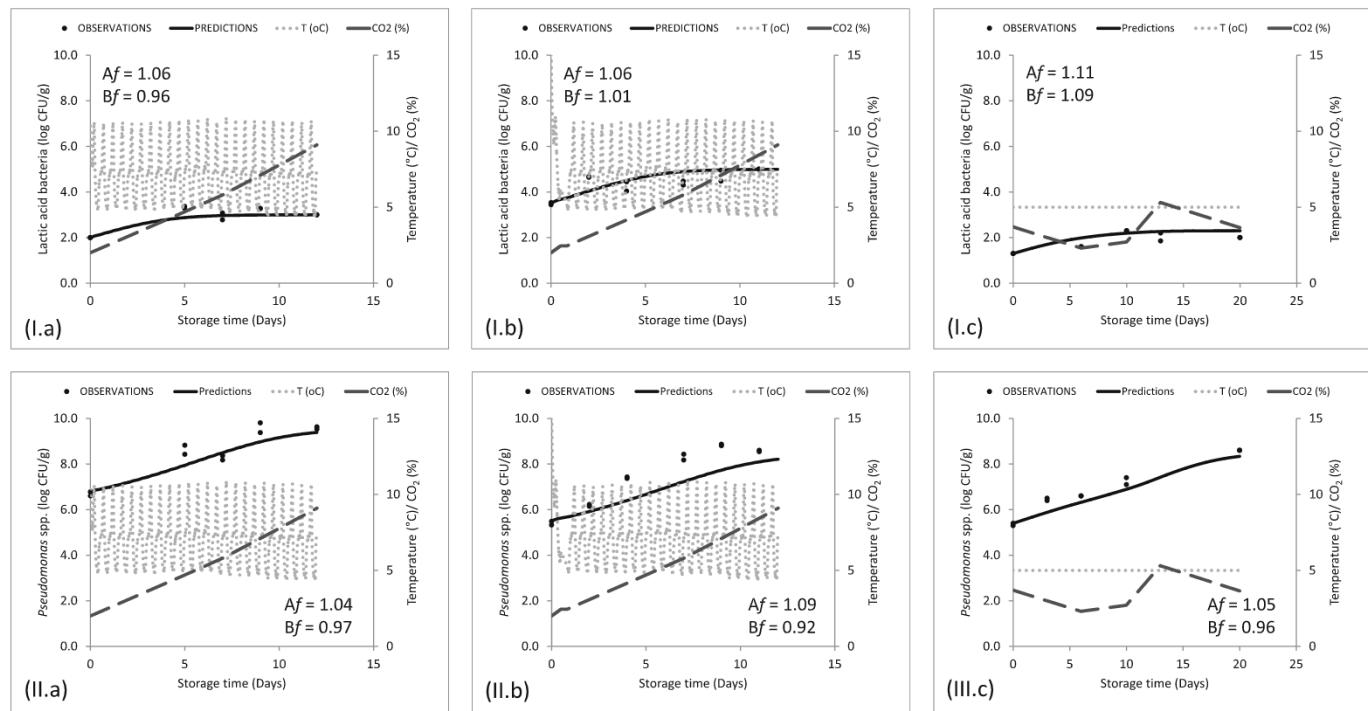


Fig. 7. Simulated growth curves and observed data of (I) LAB and (II) pseudomonads in commercially RTE fresh-cut salads (a) rocket (80 g/package), (b) romaine lettuce (200 g/package), and (c) iceberg lettuce (200 g/package) packaged under 3% O₂: 10% CO₂: 87% N₂ in laser micro-perforated commercial packages and stored at dynamic temperature (8 h at 8 °C, 8 h at 12 °C and 8 h at 4 °C) and isothermal temperature of 5 °C.

(Fig. 3(III.a) – (III.d)). The latter result indicates that the respiration rate was not eliminated, even though destruction of rocket tissue was performed; however still, gases variation in the headspace was lower than on rocket salad (Fig. 2(III.a) – (III.d)). Moreover, the initial concentration of gases remained stable for an extended storage period when rocket pulp was packaged under 15% O₂: 5% CO₂: 80% N₂ at all assays (Fig. 3(IV.a) – (IV.d)). The packaging atmosphere of 20% O₂: 0% CO₂: 80% N₂ (i.e., close to air composition) showed high stability in headspace gas concentration, except for storage at 15 °C, where the O₂ decreased from 20.08 to 15.45% within the first 4 days (Fig. 3(V.a) – (V.d)). Overall, variation in the headspace gas concentration of rocket pulp samples was either eliminated or significantly decreased ($p < 0.05$) compared to rocket salad, under the different MAP conditions, thus adequately verifying the initial hypothesis of the developed vegetable – packaging model system and confirming its suitability for “isolating” the impact of temperature and % CO₂ on microbial growth.

3.2. Development of models for growth of *Pseudomonas* spp. and LAB

The changes in relative levels of spoilage association on rocket pulp were dependent ($p < 0.05$) on MAP conditions and storage temperature (Fig. 4). *Pseudomonas* spp. was the dominant spoilage microorganism in rocket pulp, regardless of storage temperature and MAP. Previous studies have reported pseudomonads as the most prevalent genus in leafy vegetables such as spinach, lollo rosso lettuce, lollo verde, and rocket (Lopez-Velasco, Welbaum, Boyer, Mane, & Ponder, 2011; Tsironi et al., 2017). The initial average population of pseudomonads (N_0) ranged from 5.80 ± 0.05 to 6.85 ± 0.09 log CFU/g, while the recorded growth rates (μ_{\max}) significantly increased ($p < 0.05$) with storage temperature from 0 °C to 15 °C, regardless of MAP (Fig. 4(I.a) – (I.e); Table 3). Increase in storage temperature had a positive effect ($p < 0.05$) also on the maximum population (N_{\max}) of pseudomonads, especially under 15% O₂: 5% CO₂ and 20% O₂: 0% CO₂ (Table 3) with maximum observed populations from 7.59 ± 0.00 to 9.41 ± 0.03 log CFU/g, depending on temperature and MAP. In fact, pseudomonads clearly dominated at 0 °C, whereas co-dominated with

LAB at 10 °C and 15 °C, even when rocket pulp was packaged under 15 or 20% CO₂, indicating that their high initial populations was sufficient to overcome the limited O₂ available in the packaging atmosphere (Fig. 2(I.a) – (I.d) and (II.a) – (II.d)). On the other hand, LAB had the lowest recorded initial populations of 2.69–3.78 log CFU/g, among the studied microorganisms. Their growth however, was significantly favored ($p < 0.05$) by the increase in storage temperature and CO₂ levels (Fig. 4; Table 2). Specifically, when rocket pulp was packaged under 15 and 20% CO₂ and stored at 10 and 15 °C, LAB manage to quickly co-dominate with pseudomonads (Fig. 4(I.c), (I.d), (II.c), and (II.d)). The positive effect of % CO₂ on LAB growth is strongly related to their microaerophilic nature along with the limited variation of gases concentration inside the package ($p \geq 0.05$), as mentioned before and shown in Fig. 3(I.a) – (I.d) and (II.a) – (II.d). Growth potential of LAB was limited at temperatures close to the recommended for fresh-cut vegetable salads (0 and 5 °C) and as % O₂ increased in the packaging atmosphere (Fig. 4(IV.a), (IV.b), (V.a), and (V.b)). Under the latter conditions, enterobacteria along with yeasts and molds outcompeted LAB, but at significantly lower levels than pseudomonads (Fig. 4(IV.a), (IV.b), (V.a), and (V.b)).

Growth of pseudomonads and LAB was selected for modelling due to their dominance and high growth potential (Tables 2 and 3; Fig. 5), using the classical two stage approach: first, a primary model was fitted to the growth curves, estimating the growth kinetics (N_0 (log CFU/g); N_{\max} (log CFU/g); μ_{\max} (days⁻¹); λ (days)) of the two organisms in rocket pulp, followed by a secondary model (polynomial) describing the dependence of the growth rate on temperature and CO₂ concentration in the package. The parameters and statistics of polynomial model for the growth of both microorganisms are illustrated in Table 4. The secondary models for $\ln(\mu_{\max})$ of LAB and pseudomonads showed R^2_{adj} of 0.975 and 0.840, respectively (Table 4).

3.3. Performance of the growth models in comparison to experiments in commercial packages of fresh cut leafy vegetables

Growth models based on the isothermal experiments for

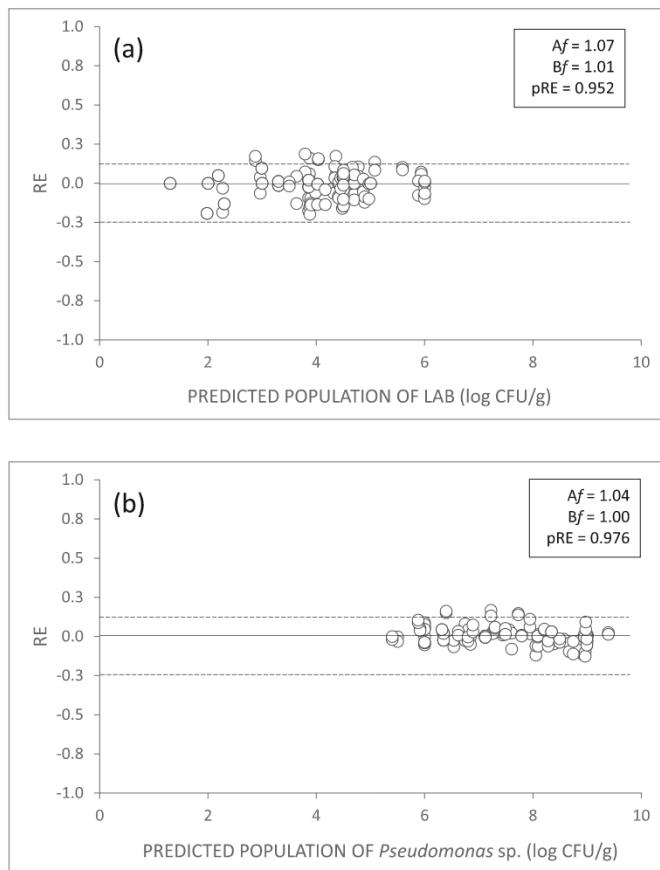


Fig. 8. Relative error (RE) plots with an acceptable prediction zone (APZ) from an RE of -0.3 (under-predictions) to 0.15 (over-predictions) for comparison of observed and predicted values of (a) LAB and (b) pseudomonads populations ($\log \text{CFU/g}$), taking into account independent data from all validation experiments.

Table 4

Estimated parameters of secondary polynomial models for $\ln(\mu_{\max})$ and fitting performance (R^2_{adj} ; RMSE) for the growth of lactic acid bacteria and pseudomonads in rocket pulp packaged under different MAP (0% O_2 : 20% CO_2 ; 5% O_2 : 15% CO_2 ; 10% O_2 : 10% CO_2 ; 15% O_2 : 5% CO_2 ; 20% O_2 : 0% CO_2) and stored at 0, 5, 10, and 15 °C.

Parameter	LAB		<i>Pseudomonas</i> spp.	
	$\ln(\mu_{\max})$ (day^{-1})	p-value	$\ln(\mu_{\max})$ (day^{-1})	p-value
a_0	-1.656 ± 0.066	0.000	-2.738 ± 0.519	0.000
CO_2	0.003 ± 0.011	0.538	0.403 ± 0.084	0.000
T	0.205 ± 0.014	0.000	0.186 ± 0.106	0.044
CO_2^2	0.000 ± 0.000	0.554	-0.030 ± 0.004	0.000
T^2	-0.004 ± 0.001	0.818	-0.001 ± 0.006	0.638
$T^* CO_2$	0.000 ± 0.001	0.835	-0.008 ± 0.004	0.179
R^2_{adj}	0.975		0.840	
RMSE	0.127		0.997	

Coefficients with p-value < 0.05 were statistically significant.

pseudomonads and LAB were validated against various commercially available fresh-cut vegetables (rocket, romaine lettuce, and iceberg lettuce), MAPs, packaging films (low and high permeability), and storage temperature conditions (isothermal and dynamic) (Table 1; Figs. 5–7). The recorded values of bias factor ranged from 0.92 to 1.12 per validation experiment, indicating no substantial bias of the models (Figs. 5–7). Moreover, accuracy factors showed good agreement between predictions and observations ranging from 1.03 to 1.11 for all validation experiments. Values of Af , Bf , and pRE were calculated also

for a pooled data set of all validation experiments (Fig. 8), in order to provide a collective overview for the performance of the two developed models. Specifically for pRE index, 120 of 126 REs ($pRE = 0.952$) and 123 of 126 REs ($pRE = 0.976$) were inside the acceptable prediction zone (-0.3 to 0.15) ((Fig. 8(a) and (b)), for LAB and pseudomonads populations, respectively. Thus, both polynomial models had acceptable performance, since they met the criterion of $pRE \geq 0.70$ (Oscar, 2005), along with presenting Bf of 0.99 (LAB) or 1.00 (pseudomonads) and Af of 1.07 (LAB) or 1.04 (pseudomonads). Overall, a good agreement between predictions and observations was obtained for all validation experiments, suggesting that the two generic models based on rocket pulp may be applicable in predicting pseudomonads and LAB in commercial packages of a variety of fresh-cut salads such as romaine and iceberg lettuce, regardless of botanical type, tissue post-harvest physiology and packaging characteristics.

4. Conclusions

The fresh-cut salads industry is expected to continue expanding rapidly in the forthcoming years, enforcing the need for improved methods of reliable shelf-life estimations, such as predictive modelling. Identifying that the existing secondary models for fresh-cut salads are product and packaging specific (García-Gimeno & Zurera-Cosano, 1997; Sinigaglia et al., 1999; Tsironi et al., 2017), the present research aimed to minimize critical parameters such as film permeability, respiration rate of vegetable, free volume inside the packaging in order to enable the collection of modelling data that quantify the impact solely of temperature and % CO_2 on growth of pseudomonads and LAB. Considering that a high number of validation experiments were performed, the capacity of the developed vegetable – packaging model system to be used as basis for (generic) modelling of microbial growth in various fresh cut salads was confirmed for a variety of commercial fresh-cut salads such as rocket, romaine lettuce, and iceberg lettuce; however further experimentation and validation trials with fresh-cut vegetables of moderate or low respiration rate such as cabbage, carrot, onion need to be performed.

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