Article

Microbiological Quality of Ready-to-Eat Salads During Shelf Life and Home Refrigeration

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Abstract: The market of ready-to-eat salads is experiencing a noticeable growth in Europe. Since they are intended to be consumed without additional treatments, these ready-to-eat products are associated with a high microbiological risk. The aim of this work was to evaluate the microbiological quality and safety of ready-to-eat salads sold in widespread supermarket chains in Lazio, Italy, at the packaging date, during shelf-life and during home-refrigeration. The study also aimed to determine the differences between low, medium, and high cost products. *Salmonella* spp., *L. monocytogenes* were chosen as safety indicators as specified by European regulations while total aerobic mesophilic bacteria and *Escherichia coli* were chosen as quality indicators as suggested by national guidelines. Analyses were performed following the ISO standards and in parallel, for the evaluation of total aerobic mesophilic bacteria, with an alternative colorimetric system, the Micro Biological Survey method, in order to propose a simple, affordable and accurate alternative for testing the microbiological quality of products, especially suitable for small and medium enterprises and on-site analyses. The study revealed high, unsatisfactory, total bacterial loads in all analyzed samples at the packaging date and expiring date and a very high prevalence of *Salmonella* spp. (67%) regardless of the selected varieties and cost-categories; *L. monocytogenes* was instead not recovered aligning with the results obtained in other studies.

Keywords: RTE salads; Microbiological quality; shelf-life; MBS method

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1. Introduction

Industrialized countries have recently faced an emerging demand for healthy and time-saving dietary solutions consistent with the modification in eating habits and the reduced time available for food preparation [1-2]. In particular, the consumption of ready-to-eat salads (RTES) has experienced a noticeable increase in Europe and especially in Italy, where, following a 10% average annual increase, 2% of the vegetable market is involved in the production of RTE vegetables, reaching a turnover of about 600 million Euros [3, 4].

The commercial success of these products is linked to the explicit and implicit services they offer: fresh, safe, healthy and nutritionally valuable products that can be consumed without preparation time are appealing to consumers who desire to improve their diet and save time [5]. Moreover, RTES are 100% edible and are socially perceived as very high-quality products [6, 7].

RTE food products are minimally processed products intended to be consumed without additional treatments. RTE leafy green vegetable processing includes several steps: after a first selection and elimination of external wilted or ruined leaves, the selected leaves are cut, washed, dried and packed in plastic containers [8, 9]. The minimal technological processing ensures the preservation of organoleptic properties but is related to a generally shorter shelf-life compared to the

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starting product. The average shelf-life of RTES ranges from 5 to 7 days, and, after packages have been opened, products can be stored at refrigeration temperatures lower than 8°C for maximum 2 days. Modified atmosphere packaging (MAP) has been introduced as an upgrading technology to extend shelf-life and is currently adopted by major industries but still not always implementable in small and medium-sized enterprises (SMEs), that represent an important market sector in many countries, including Italy [10].

The main issue associate to these products is the high microbiological risk associated with their consumption. Microbiological contamination is common and inevitable in vegetables growing in soil. Typical environmental microorganisms found in soil and irrigation water contaminate plants infiltrating through roots or exposed (wounded or cut) surfaces and get internalized by the plant's coating that creates a natural biofilm that protects them from surface treatments. The microflora can be further modified by other microorganisms that come in contact with the product during each step of the production chain [11-16].

RTES are in fact involved in the transmission of foodborne pathogens: the high moisture content, the permissive pH (6.0-7.0), the lack of stringent decontamination procedures and the impact of temperature abuse during processing, transportation and storage can further increase the risk associated to these products. The number of gastroenteritis cases associated to RTE vegetables consumption has been increasing in the last years [17-20] and several outbreaks have been connected to the consumption of salads contaminated by *Salmonella* spp. [21], *Listeria monocytogenes* [22, 23] and *Escherichia coli* O157: H7 [24, 5]. Furthermore, RTES may have an important role in the spread of bacteria of clinical interest carrying antibiotic resistance genes [25, 26].

According to European Regulation (EC) No 1441/2007 the absence of *Salmonella* spp. and concentrations of *L. monocytogenes* lower than 100 colony forming units (CFU)/g are considered essential criteria to define safety of RTES placed on the market during their shelf-life. Although no mandatory microbiological criteria include the evaluation of total aerobic mesophilic count (TAMC) or *E. coli*, several guidelines include these parameters as indicators of the overall microbiological quality of RTE foods' production processes [27, 28]. High concentration levels could be indicator of an inadequate treatment a lowered shelf life and an overall higher microbiological risk. In particular, according to Portuguese guidelines [29], for RTE salads TAMC and *E. coli* satisfactory, acceptable and not acceptable levels are specified (Table 1).

Table 1. Satisfactory, acceptable and not acceptable total aerobic mesophilic count (TAMC) and *E. coli* contamination levels (colony forming units per gram) for ready-to-eat salads according to Portuguese guidelines [29].

Indicators	**Contamination levels (CFU/g)**		
	Satisfactory	Acceptable	Not acceptable
TAMC	$\leq 10^4$	$>10^4 \leq 10^6$	$>10^6$
E. coli	≤ 10	$>10 \leq 10^2$	$>10^2$

In the last years several research groups have studied the microbiological quality of RTES highlighting high counts of total aerobic mesophilic count, coliforms, yeasts and molds but no presence of *Salmonella* spp. and *L. monocytogenes* [30-40]. Less attention has instead been addressed to the evolution of microflora during shelf life and during home-refrigeration after package opening.

The aim of this work is to evaluate the microbiological quality and safety of RTES sold in widespread supermarket chains in Lazio, Italy, at the packaging date, during shelf-life and during home-refrigeration. The study also aims to determine the differences between low, medium and high cost RTES and the impact of MAP technology in terms of quality and safety of these products. *Salmonella* spp., *L. monocytogenes* were chosen as indicators of RTES safety while TAMC and *E. coli* as indicator of RTES quality. Pathogens detection was performed following reference ISO methods as required by EU Regulation while TAMC was performed according to reference ISO and the alternative Micro Biological Survey (MBS) method. The MBS method is a colorimetric system for easy detection and the selective count of bacteria present in agro-food in water and in environmental

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samples [41], developed, produced, and commercialized by MBS srl, 00131 Rome (Italy), a former spin-off company of Roma Tre University. The method, that has already demonstrated to efficiently carry out microbiological analyses [42-44], and its accuracy and repeatability in comparison to the reference method for TAMC has been largely demonstrated in previous works [41, 45].

2. Materials and Methods

2.1 Evaluation of RTES microbiological quality and safety during shelf-life.

Samples. Two different varieties of RTES were selected among the products commercially available in Italian supermarkets: baby romaine lettuce (BRL) and rocket salad (RS). Varieties were chosen from a low-cost (LC) brand, sold in a popular discount supermarket, a medium-cost (MC) store-brand, sold in a higher-priced supermarket, and a high-cost (HC) top-selling brand-name. A total of 6 production batches for each variety and each category, were analyzed at the packaging date and at the expiring date (total batches=36). Two bags from the same batch were purchased on the packaging date and transported in their primary package and under refrigeration conditions (4±1°C) to the laboratory. One was immediately analyzed, and the other one was stored at 4°C, opened, and analyzed on the expiring date.

Sample preparation. Samples were prepared homogenizing 30 g in 275 ml of Buffered Peptone Water (BPW, Applichem, Darmsdadt, Germany) using a Stomacher 400, Seward, London, UK for 120 s at medium speed and serially diluted in the same diluent when needed.

Evaluation of TAMC using the pour plate method. Evaluation of TAMC was performed according to UNI EN ISO 4833-1:2013. Samples were prepared as previously described. One ml of the selected dilutions was transferred into the Petri dishes in triplicate, then 15 to 17 ml of Plate Count Agar (PCA) medium (Applichem, Darmsdadt, Germany) at 45°C was poured into each Petri dish. Plates were inverted and incubated at 30°C for 72 h. Colonies in plates with 25 to 250 colonies were counted and viable counts in the test sample per gram were calculated as follows:

$$N = \sum C / [n_1 + 0.1 n_2)] \times d$$

where: N = number of colonies per ml or gram of sample.

$\sum C$ = sum of all of the colonies in all plates counted.

n_1 = number of plates in the lower dilution counted.

n_2 = number of plates in the next higher dilution counted.

d = dilution factor corresponding to the first dilution retained

Evaluation of *E. coli* using the pour plate method. Evaluation of beta-glucuronidase-positive *E. coli* was performed according to UNI EN ISO 16649-2:2010. Samples were prepared as previously described. One ml of the selected dilutions was transferred into the Petri dishes in triplicate, then 15 to 17 ml of Tryptone Bile -glucuronide (TBX) agar medium (Applichem, Darmsdadt, Germany) at 45°C was poured into each Petri dish. Plates were inverted and incubated at 44°C for 24 h. Colonies displaying the typical morphological characteristics (blue to blue-green) in plates containing 15-150 typical CFU and less than 300 total (typical and non-typical) CFU were counted and the number of CFU of β-glucuronidase-positive *E. coli* present in the test sample per gram were calculated as follows:

$$N = \sum a / [(n_1 + 0{,}1 n_2)] \times d$$

where: N = number of colonies per ml or gram of sample.

$\sum a$ = sum of the CFU counted on all the dishes retained from two successive dilutions, at least one of which contains a minimum 15 blue CFU.

n_1 = number of plates in the lower dilution counted.

n_2 = number of plates in the next higher dilution counted.

d = dilution factor corresponding to the first dilution retained

Evaluation of TAMC using the MBS method. The MBS method is a colorimetric system for detection and quantification of bacteria in food and water samples. TAMC using the MBS method was performed using MBS Total Viable Count (TVC) vials, containing the specific lyophilized growth medium for the detection and quantification of viable mesophilic aerobic bacteria.

To start the analysis, vials were rehydrated with 10 mL of sterile distilled water and paraffin oil, and shaken until all the reagent was dissolved. Vials were inoculated with 1 mL of samples homogenate and its serial dilutions, in parallel with the reference pour plate method. All analyses were performed in triplicate. Vials were incubated at 30°C for 30 h.

The vials' medium color was periodically controlled with a thermostatic colorimeter that automatically detects the color change. A color change from blue to yellow of the reaction medium is indicative of a positive result, i.e. the presence of aerobic mesophilic bacteria [45]. The time for color change after inoculum varies according to the bacterial concentration. The time for color change was inversely related to the bacterial content of the analyzed sample: the higher the bacterial concentration, the less the time required for color change. The persistence of the starting color indicates a negative result; that is, absence of the microorganisms of interest. Regression lines were obtained plotting the time taken for the TVC vials to change color against the logarithm of the TAMC concentration obtained with the reference method.

Detection of pathogens of interest. Recovery of the pathogens of interest according to Regulation (EC) No 1441/2007 was performed according to UNI EN ISO 11290-2:2017 and UNI EN ISO 6579-1: 2017 respectively for enumeration of *L. monocytogenes* and for detection of *Salmonella* spp. Both analyses were performed on the same food homogenate.

For the enumeration of *L. monocytogenes,* the food homogenate was left 1 hour at room temperature and then 1 ml was plated in 3 PALCAM agar plates in duplicate and incubated at 37°C for up to 48 h. Gray-green colonies surrounded by dark brown to black halos were cultured in BHI broth overnight at 37°C and the confirmation was performed on using the qualitative immunoassay for the determination of *Listeria monocytogenes* (LISTERIA M. CARD, InterMedical, Villaricca, NA, Italy).

For the detection of *Salmonella* spp., the food homogenate was incubated at 37°C for 24 h. After this pre-enrichment step, 1 ml and 100 µl of the pre-enrichment broth were transferred respectively in 10ml of Muller Kauffmann Tetrathionate Broth and Rappaport Vassiliadis broth and incubated respectively at 37°C and 44°C for 24 h. Next, 10 µl of the two selective broths were spread in duplicate on XLD and BGA agar plates and incubated at 37°C for 24 h. Five colonies (or all if < 5 CFU) displaying the typical morphological *Salmonella* characteristics (pinkish red colonies on BGA and red colonies with black centers) were cultured in BHI broth overnight at 37°C and the confirmation was performed using the qualitative immunoassay for the determination of *Salmonella* spp. (SALMONELLA Ag CARD, InterMedical, Villaricca NA, Italy). Additionally, 5 non-suspected colonies underwent confirmation following the same procedure.

2.2 Evaluation of RTES microbiological quality simulating home refrigeration after package opening.

Samples. Three different varieties of RTES were selected among the products commercially available in Italian supermarkets: BRL, RS and lamb's lettuce (LL). Varieties were chosen from a LC brand and a HC top-selling brand. A total of 3 production batches for each variety and each category were selected (total number of batches=18). The bags were purchased on the packaging date, transported, in their primary package and under refrigeration conditions (4±1°C), to the laboratory and analyzed on the packaging date. The opened bags were re-sealed and analyzed after a 2-days storage at 4°C, as per manufacturer indication, simulating home-refrigeration conditions.

Sample preparation. Samples were prepared homogenizing 30 g in 275 ml of Buffered Peptone Water (BPW) using a Stomacher 400, Seward, London, UK for 120 s at medium speed and serially diluted in the same diluent.

Evaluation of TAMC using the pour plate method. Evaluation of TAMC according to ISO 4833-1:2013 was performed as previously described.

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Evaluation of TAMC using the MBS method. Evaluation of TAMC according to MBS method was performed as previously described.

Statistical analysis. Statistical analysis of variance (ANOVA) and covariance (ANCOVA) was performed using Past (Paleontological Statistics package for education and data analysis) version 3.12 for Windows.

3. Results

3.1. Evaluation of RTES microbiological quality and safety during shelf-life.

BRL and RS samples were selected among the products commercially available in Italian supermarkets. The microbiological quality and safety of RTES was evaluated during shelf-life: the enumeration of *L. monocytogenes,* the detection of *Salmonella* spp., TAMC and evaluation of *E. coli* were performed on packaging and expiring date. Table 2 shows the results obtained for pathogen recovery: all batches resulted compliant to European standards for *L. monocytogenes*; conversely 67% of the analyzed batches resulted positive for *Salmonella* spp. resulting not compliant to European regulations. All the *Salmonella* positive batches were found to be positive both on the packaging and the expiring date.

Table 2. Ready-to-eat salad batches acceptability according to European Regulation (EC) No 1441/2007 safety criteria: for *Salmonella* spp. absence in 25 grams, for *L. monocytogenes* lower than 100 colony forming units (CFU)/g.

	***Salmonella* spp.**	***L. monocytogenes***
Compliant	12	36
Non compliant	24	0
Total	36	36

TAMC results for BRL and RS are displayed in Figure 1. Of all the samples analyzed only 17% displayed an acceptable level of contamination according to Portuguese guidelines (10^4< CFU/g <10^5).

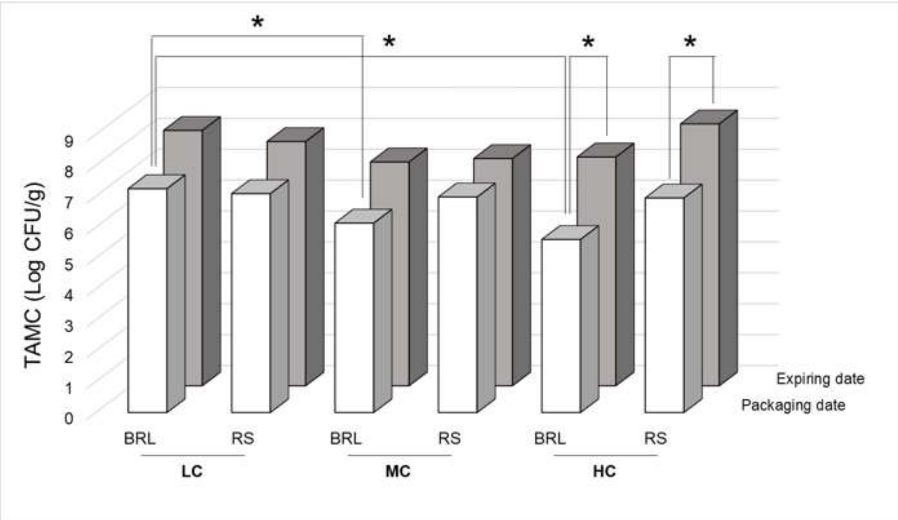

Figure 1. Ready-to-eat salads average total aerobic mesophilic count (TAMC) contamination levels at packaging and expiring date for low cost (LC), medium cost (MC) and high cost (HC) baby romaine lettuce (BRL) and rocket salad (RS) samples (SD<10%) evaluated using the plate count method

* Significant difference (P< 0,05)

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The average concentration at the packaging date was of 6.63 (±0.64) and 7.63 (±0.42) Log CFU/g respectively with an average growth of 1 Log unit (+15%) at the expiring date. At the packaging date 100 %, 67% and 33% samples of BRL displayed unsatisfactory results (TAMC > 10^6 CFU/g) for LC, MC and HC respectively. For RS, at the packaging date 100% of LC and MC and 83% of HC samples resulted unsatisfactory (TAMC > 10^6 CFU/g). At the expiring date, instead, all samples, independently of the variety and the cost category displayed unsatisfactory results. A significant difference at the packaging date in TAMC for BRL was observed between LC and MC and between LC and HC samples; no significant difference was instead observed for RS samples. A significant difference between TAMC at the packaging date and expiring date was observed only for HC samples both for BRL and RS. The overall percent increase in growth for each variety and each cost category are displayed in Table 3.

Table 3. Ready-to-eat salad average total aerobic mesophilic count (TAMC) percent increase in growth from packaging to expiring date for each variety and cost category.

	LC	MC	HC
Baby Romaine lettuce	+11%	+18%	+29%
Rocket salad	+10%	+5%	+20%
Overall	+10,5%	+11,5	+24,5%

All samples resulted instead acceptable regarding the presence of *E. coli* that was recovered in only 1 batch of MC rocket salad in concentration < 100 CFU/g.

3.2. Evaluation of RTES microbiological quality simulating home refrigeration after package opening.

Baby romaine lettuce, rocket salad and lamb's lettuce samples were selected among the products commercially available in Italian supermarkets. The percent increase in growth after package opening, simulating home-refrigeration was evaluated at the packaging date and after 2 days of storage of the open bags at 4°C.The TAMC results for BRL, RS and LL are displayed in Figure 2 (2a, 2b and 2c respectively). At the packaging date 100% and 33% samples of the analyzed batches displayed unsatisfactory results (TAMC > 10^6 CFU/g) for LC and HC respectively. The average concentration at the packaging and expiring date was of 6,96 (±0,55) and 7,45 (±0,56) Log CFU/g respectively with an overall growth of 0,5 Log unit (+7%).

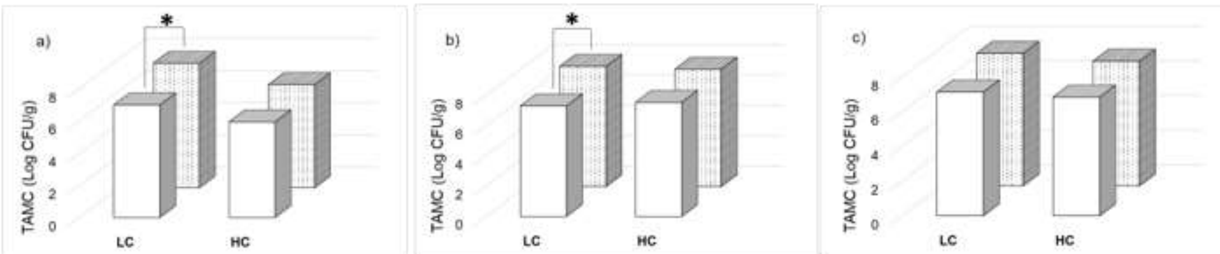

Figure 2. Ready-to-eat salad average total aerobic mesophilic count (TAMC) contamination levels at packaging date (☐) and after 2 days of storage of the opened bags at 4°C ( ) for low cost (LC) and high cost (HC) samples: 2a baby romaine lettuce (BRL); 2b rocket salad (RS); 2c lamb's lettuce (LL) samples (SD <10%) evaluated using the plate count method * *Significant difference (P< 0,05).

After 2 days from opening all the analyzed samples displayed a TAMC concentration > 10^6 CFU/g. A significant difference between TAMC at the packaging date and after 2 days of storage of the opened bags at 4°C was observed only for LC BRL and RS samples; no significant difference was instead observed for

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HC samples independently from the variety. The overall percent increase in growth for each variety and each cost category are displayed in Table 4.

Table 4. Ready-to-eat salad average total aerobic mesophilic count (TAMC) percent increase in growth after packaging opening during a simulated home refrigeration of 2 days at 4°C for each variety and cost category.

	LC	HC
Baby romaine lettuce	+11%	+8%
Rocket salad	+9%	+3%
Lamb's lettuce	+7%	+5%
Overall	+9%	+5.3%

3.3. Accuracy of the MBS method

TAMC analyses were performed with the reference method and the alternative MBS method. Linearity of the MBS method was evaluated according to ISO 16140:2016. Correlation between the time taken for the MBS TVC vials to change color and the log CFU/ml of TAMC is shown in Figure 3. A linear inverse relationship between the time for color change of the MBS TVC vials and the TAMC at 30°C (log CFU/g) was observed (slope= 0,30; maximum analysis time= 30 hours; R^2=0,79) (Figure 3).

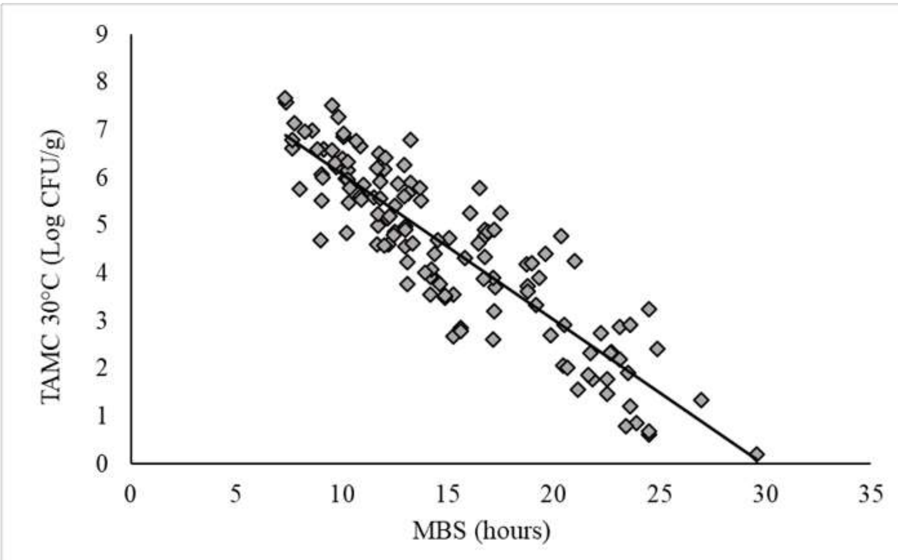

Figure 3. Correlation line between the results obtained with the MBS method and reference methods. The total aerobic mesophilic count (TAMC) quantitative results obtained with reference method were plotted against the time taken for the MBS Total Viable Count vials to change color. Continuous line represents the linear regression analysis (slope = 0.30, R^2 = 0.79). Each analysis was performed in triplicate (SD < 0.4).

4. Discussion

In this work we have analyzed the microbiological quality and safety of RTES sold in Lazio, Italy, taking into consideration different varieties and cost-categories.

With regard to pathogens, interestingly, the prevalence of *Salmonella* spp. was very high (67%) and no significant difference could be observed among the selected varieties and cost-categories; *L. monocytogenes* was instead not recovered aligning with the results obtained in other studies [34-38] The divergence between the results obtained in this work from those obtained by other groups regarding the presence of *Salmonella* spp. could be explained by the fact that most of the positive results following immunological confirmation were obtained from non-suspected colonies, displaying non typical morphological characteristics.

The study of TAMC during shelf-life revealed many unsatisfactory results: at the packaging date HC batches resulted less contaminated compared to LC and MC probably linked to the specific packaging conditions (MAP). At the expiring date instead, all batches displayed unsatisfactory results: an average higher percent increase in growth has been observed in HC salads compared to LC and MC. We hypothesize that this could be due by the fact that the MAP condition could affect the existing microflora by reducing the initial contamination level and selecting a specific microflora that, in the unaltered MAP product environment, is advantaged by the higher availability of nutrients and the reduced competition compared to those of LC and MC. A slightly different trend was observed simulating home-refrigeration after package opening. At the packaging date HC batches resulted less contaminated compared to LC in baby romaine lettuce while no significant difference was observed for the other samples. After two days of storage at 4°C of the opened bags all the analyzed samples displayed unsatisfactory results; the growth trends were similar to those observed during shelf-life for LC batches while they were considerably reduced for HC batches. This difference may be indicative of the fact that the altered MAP environmental condition caused by the bags' opening could in some way affect the metabolism of resident microflora.

TAMC were performed in parallel with the MBS method with satisfying outcomes. The MBS method resulted quite accurate (R^2= 0, 78) and was able to detect <5 CFU/ml in 30 hours significantly reducing the standard analytical times (72 hours). The simple procedure, the simplified interpretation of results and the stand-alone equipment could be very useful tool to streamline microbiological analysis particularly in small, medium enterprises.

In conclusion, the microbiological quality of RTES seems to be still a challenge despite the advances in technology and the attention from Regulations and International food safety agencies. The obtained results highlight the need for a more extensive microbiological control and suggest optimization of large-scale washing procedures. A more attentive analysis of the possible conditions occurring during shelf-life and domestic storage should be also considered. The implementation of an accurate, fast, easy and portable microbiological method of analysis could be a valuable tool to provide higher quality products.

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Conflicts of Interest: The authors declare no conflict of interest

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