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Temporal pattern of microbial indicators of ready-to-eat rocket salads during shelf life

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Abstract

Introduction. From 2001-2009 there have been numerous community alerts and notifications about the rocket salad produced in Italy and distributed in Europe. Our study describes the evolution of the microbial quality of ready to eat rocket salad during shelf life among three different Italian producers.

Material and methods. Total Mesophilic Count (TMC) and *Escherichia coli* (EC) count were measured in 248 samples. We used Wilcoxon test to compare the median values of TMC and EC counts and Kruskal Wallis test to compare the producers.

Results. The TMC and EC values differed among producers at the stages of raw material and in the finished product (Kruskall Wallis test, $p < 0.05$). The evolution of bacterial charges had significant differences among producers at expiration date (Wilcoxon test, $p < 0.05$). More than half of the samples (54.8%) exceed reference standard for TMC after 48 h from packaging.

Conclusion. Differences among producers may linked to the different minimal processing technologies adopted after harvesting.

Key words
- microbial trend
- ready-to-eat vegetables
- minimal processing

INTRODUCTION

The recent advancement of agronomic practices coupled with the development of efficient technologies for the processing, preservation, packaging and distribution of raw vegetables permit the fresh vegetable industry to continuously supply consumers with a wide range of high-quality products in most countries of the world. Raw and minimally processed vegetables are those available to the consumer in a ready-to-eat (RTE) form and are defined as a product that has been peeled, cut or otherwise physically altered from its original form, but remains in a fresh state and is intended for consumption raw [1]. In Europe, the increasing demand of fresh vegetables is causing an expansion of the market share of RTE vegetables. For example in Italy, the consumption of RTE vegetables has shown a substantial growth from 14,736 t (corresponding to a market value of 138.677 million euro) in 2002 to 50,723 t (397.599 million euro) in 2009.

The increased consumption of these products implies an increased risk for health of consumers, because several pathogenic microorganisms can be found in vegetables, including Verocytotoxin-producing *Escherichia coli*, *Salmonella enterica* and *Listeria monocitogenes* [2-6]. In recent years, a growing number of foodborne disease outbreaks have been traced back to the consumption of raw vegetables [7-16] increasing the concern that vegetables might be more important as vehicle for pathogens than previously thought.

The contamination of RTE products may take place along the whole production chain. Potential sources of contaminants include the water used for irrigation and in all the phases of production chain after the harvest, the microbiological quality of soil and the cross-contamination from the staff handling the products [17].

In recent years, the European Rapid Alert System for Food and Feed (RASFF) has repeatedly drawn the attention of Member States on the microbial contamination of vegetables products. In particular, in the period 2001-2009 there have been numerous community alerts and notifications about the rocket salad (*Eruca sativa* also known as arugula or rucola lettuce) produced in Italy and distributed in several European Countries. Most of these alerts originated in Sweden and were related to *Salmonella* (*S. Napoli, S. Thompson, S. typhimurium*) contamination in rocket salad imported from Italy

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but other alerts for the same reason were reported also in Norway, in England, in Finland, and Slovenia [12] (https://webgate.ec.europa.eu/rasff-window/portal/). These repeated community alerts and notifications require the acquisition of scientific evidence about the pre and post harvest determinants of the microbial quality of Italian RTE rocket salad. However, we are still lacking of basic knowledge on the dynamics of common microbial indicators during the shelf life of this product and their variation among different producer brands.

The aim of this study is to assess the evolution through time of microbiological counts of RTE rocket salad in Italy during shelf life and to assess the variation among different producers. We selected Total Mesophilic Count (TMC) and *Escherichia coli* (EC) as major general indicators. Since different producers may use different minimal processing technologies (post harvest technologies), we estimate the variability of microbial counts through time among three different producers.

MATERIAL AND METHODS

RTE rocket salad production line of studied producers

All producers, located in central Italy, follow the application of HACCP (Hazard Analysis and Critical Control Point) [17]. In addition to a strict application of HACCP, Producer 1 follows also Good Agricultural Practice (GAP). Producer 2 and 3 basically differ from Producer 1 for a less stringent application of HACCP, no rigorous application of HACCP and different approaches to microbial reduction (use of chlorinated water for Producer 2, or rapid refrigeration before packaging Producer 3; see below). Temperatures during the processing in factory are maintained at 10 ± 2 °C for Producer 1 and 3; 15 °C for Producer 2. The phases of post harvest production of RTE rocket salad of the three producers are briefly described below:

1. Field harvesting. Only Producer 1 undertakes a processing stage before entering the production line. This follows GAP that includes the washing of raw rocket after harvesting in the field using water that meets the quality criteria of the *Codex Alimentarius* [18] and the discard of soil in excess.
2. Factory reception of raw materials and storage. During this stage, a macroscopic control is carried out by the staff to determine the general quality of received rocket (including an evaluation of residues of soil, stones, etc.) maintaining the temperature of rocket at 4 ± 2 °C. Storage, when necessary, is under controlled conditions of temperature (4 - 8 °C) in refrigerated storerooms, avoiding exposure to light.
3. Pre-washing and preparation. Dirty are removed from the surface of the rocket. Following that, rocket are washed a first time with potable water.
4. Washing and drying. Rocket is washed in a first washing machine to reduce the initial microbial load. The material enters afterwards a second washing machine supplemented with potable water (Producer 1 and 3) or water with the addition of only 2 ppm of free chlorine, not followed by rinsing (Producer 2). Water in excess is removed by centrifugation. At this stage, only Producer 3 executes a rapid refrigeration of the material at 4 °C at this stage using a refrigerated tunnel.
5. Packaging and labelling. Rocket is packaged in polyethylene bags in ca. 125-250 g portions and labelled with production date, expiry date, lot number, name and type of the product as well as nutritional information.
6. Storage and distribution. Packaged products are stored in refrigerated storerooms (4 ± 2 °C), avoiding exposure to light, until shipping to final destination within 24 h from packaging.

Sampling and bacterial determinations

During 2007 we obtained a total of 248 samples of raw rocket (collected just after the previously described steps 1-2) and RTE rocket (in their original sealed packages, collected just after the previously described steps 1-5). Samples (N = 92 from Producer 1, N = 108 from Producer 2 and N = 48 from Producer 3) were transported to the laboratory at 4 °C for TMC and EC determinations.

Raw rocket samples (N = 62) stored in refrigerators (4 ± 2 °C), were analysed within 48h from harvesting (time T0, corresponds to raw material before the processing stages), while sealed RTE packages (N = 186) were stored in the laboratory at 4 °C and analysed after 1 (time T1, corresponds roughly to the arrival time on shelves of commercial stores), 5-7 (time T2, corresponds to the expiration date reported on the package) and 9 days (time T3, corresponds to two days after expiration date) from packaging. The number of samples (of ca. 500 g of material each) per time and producer were as follow: N = 23 for Producer 1, N = 27 for Producer 2 and N = 12 for Producer 3. Open packages were discarded after analysis.

Bacterial determinations were carried out in double using the standard culture methods as described below: ten grams of each sample was diluted in 90 ml of Buffered Peptone Water (BPW) and homogenized for 2 minutes at 260 rpm in Stomacher (Model 400 circulator, Seward, Norfolk, England). Serial dilutions of the suspension were made in BPW and analyzed for TMC (Standard Plate Count agar) and EC (T.B.X. medium), incubated at 30 °C for 24-48 h and at 44 °C for 24h respectively, according to the standard culture methods (ISO 4833:2003; ISO 16649-2:2001), as well as the remaining steps. All microbiological media were from Oxoid (Cambridge, UK).

Statistical analysis

To study the temporal trend of microbial counts, we used the Wilcoxon signed rank test to compare the median values of TMC and EC counts at different analytical times after packaging (comparison T0-T1); at the expiration date (comparison T1-T2) and two days after expiration date (comparison T2-T3). Differences were considered statistically significant when p-values were lower than 0.05. A logarithmic transformation of the data allowed the use of a normal model for TMC but not for EC. Therefore, to increase the statistical power of the test, we included a comparison of likelihood

functions of the true log TMC values for each analytical time of the same producer.

Values of TMC and EC were compared between producers at different times with a Kruskal Wallis rank sum test followed by multiple comparisons.

For comparison with quality standards, in absence of European microbiological standards in RTE salads for TMC, we used the reference standard value of 1×10^7 cfu g^{-1} (log 7.0; limit proposed in Guidelines of PHLS [19], for ready-to-eat salads) and the European limit value of 1000 cfu g^{-1} for EC (the M value for the Hygiene process criteria of the EC Regulation n. 1441/2007 amending Regulation EC n. 2073/2005 in Europe on ready-to-eat products within the period of maximum shelf life).

RESULTS

Median TMC *(Figure 1)* differed among producers at T0 (Kruskal-Wallis chi-squared = 29.0, df = 2, p-value < 0.01); T1 (Kruskal-Wallis chi-squared = 10.8, df = 2, p-value = 0.004), T2 (Kruskal-Wallis chi-squared = 12.4, df = 2, p-value = 0.002) but not at T3 (Kruskal-Wallis chi-squared = 3.3, df = 2, p-value = 0.19). Multiple comparisons of TMC between producers at different times showed significant differences of median TMC of Producer 1 from Producer 2 at T0, T1, T2 and from Producer 3 at T2 while median TMC level of Producer 2 was statistically different from Producer 3 at T0 *(Table 1)*.

Median EC *(Figure 2)* did not differ among producers at T0 (Kruskal-Wallis chi-squared = 6.4, df = 2, p-value = 0.05) but differed at T1 (Kruskal-Wallis chi-squared = 27.8, df = 2, p-value < 0.01), T2 (Kruskal-Wallis chi-squared = 20.1, df = 2, p-value < 0.01) and T3 (Kruskal-Wallis chi-squared = 20. 6, df = 2, p-value < 0.01). Multiple comparisons of EC between producers at different times showed significant differences of median EC levels of Producer 1 from Producer 3 at T1, T2, T3 while median EC level of Producer 2 was statistically different from Producer 3 at T1, T2, T3 *(Table 1)*.

The temporal trend of TMC levels of Producer 1 *(Figure 1, upper panel)* showed a significant decrease between T0 and T1 (Wilcoxon test, V = 240, p-value = 0.001), a significant increase between T1 and T2 (Wilcoxon test, V = 43, p-value = 0.002) and a significant increase between T2 and T3 (Wilcoxon test, V = 63, p-value = 0.02137).

EC counts *(Figure 2, upper panel)* were constantly under the level of 10 cfu g^{-1} with significant differences between T0 and T1 (Wilcoxon test, V = 41, p-value = 0.03) and between T2 and T3 (Wilcoxon test, V = 6, p-value = 0.02).

The TMC levels of Producer 2 *(Figure 1, middle panel)* showed a significant decrease between T0 and T1 (Wilcoxon test, V = 369, p-value < 0.01), T1 and T2 (Wilcoxon test, V = 52, p-value < 0.01), and T2 and T3 (Wilcoxon test, V = 79, p-value = 0.02). EC counts *(Figure 2, middle panel)* were different only between T2 and T3 (Wilcoxon test, V = 25, p-value p = 0.03).

The levels of TMC of Producer 3 *(Figure 1, lower panel)* increased progressively from T0 to T2, with the only statistically significant difference between T1 and

Table 1
Multiple comparisons of Kruskal Wallis test comparing producers median Total Mesophilic Count (TMC) and *Escherichia coli* (EC) at different times

Parameter	Time	Comparison between Producers	Difference	p-value
Median TMC	T0	1-2	21.2	< 0.01
		1-3	8.4	NS
		2-3	29.7	< 0.01
Median TMC	T1	1-2	16.6	< 0.05
		1-3	5.6	NS
		2-3	10.9	NS
Median TMC	T2	1-2	14.7	< 0.05
		1-3	19.7	< 0.01
		2-3	4.9	NS
Median TMC	T3	1-2	8.8	NS
		1-3	8.3	NS
		2-3	0.6	NS
Median EC	T0	1-2	10.1	NS
		1-3	13.8	NS
		2-3	3.7	NS
Median EC	T1	1-2	11.2	NS
		1-3	32.1	< 0.01
		2-3	20.9	< 0.01
Median EC	T2	1-2	9.7	NS
		1-3	26.5	< 0.01
		2-3	16.8	< 0.05
Median EC	T3	1-2	6.2	NS
		1-3	27.7	< 0.01
		2-3	21.5	< 0.01

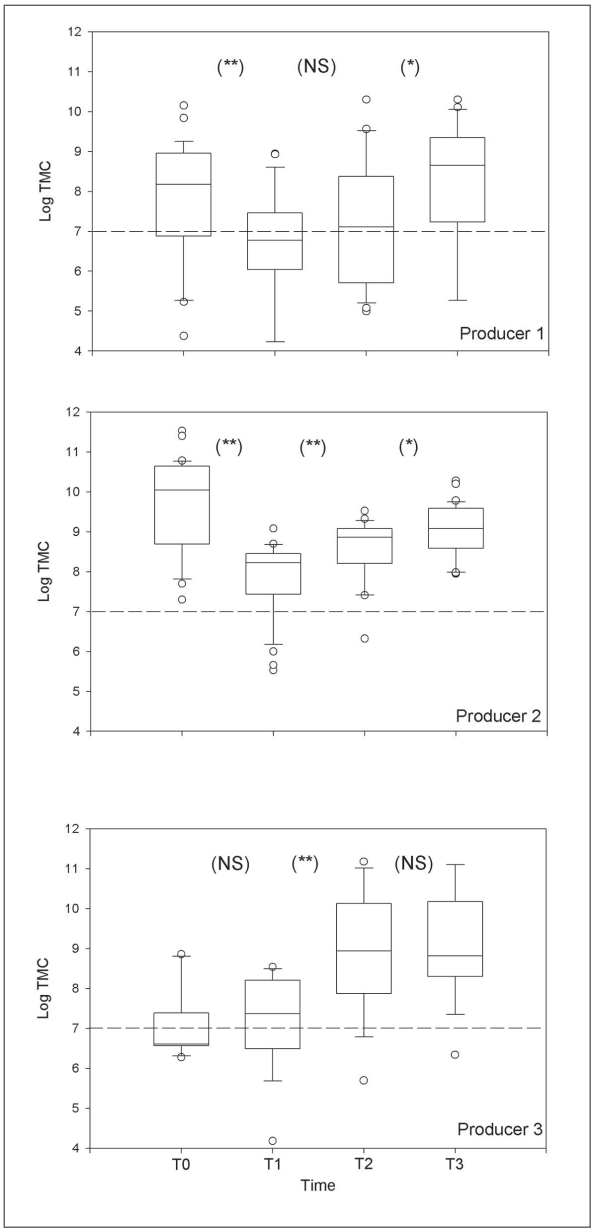

Figure 1
Box plot of log TMC values of Producer 1 (upper panel), Producer 2 (middle panel) and Producer 3 (lower panel): distribution of the log TMC values compared to TMC benchmarking log 7.0 (limit proposed in Guidelines of PHLS, 2000, for ready-to-eat salads. Significance of Wilcoxon non parametric test between analytical times after packaging is coded as follows.
(**): $p < 0.01$; (*): $p < 0.05$; NS: $p \geq 0.05$.
TMC: Total Mesophilic Count

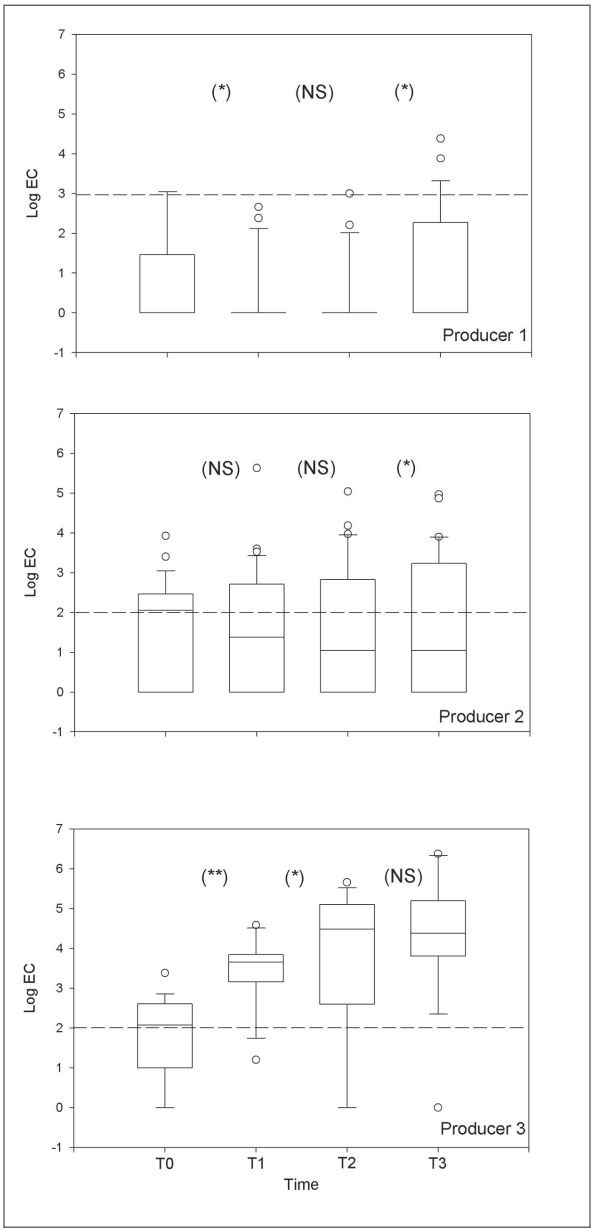

Figure 2
Box plot of log EC values of Producer 1 (upper panel), Producer 2 (middle panel) and Producer 3 (lower panel): distribution of the log EC values compared to EC benchmarking log 3.0 (limit imposed by EC Regulation n. 1441/2007). Significance of Wilcoxon non parametric test between analytical times after packaging is coded as follows.
(**): $p < 0.01$; (*): $p < 0.05$; NS: $p \geq 0.05$.
EC: *Escherichia coli*

T2 (Wilcoxon test, V = 8, p-value = 0.02) while the successive increase from T2 to T3 was not statistically significant (Wilcoxon test, V = 42, p-value ≥ 0.05). The EC counts of Producer 3 *(Figure 2, lower panel)* showed a statistically significant increase of EC from T0 to T1 (Wilcoxon test, V = 3, p-value < 0.01) and from T1 to T2 (Wilcoxon test, V = 12, p-value < 0.05).

The representation proposed in *Figure 1* shows the distribution log TMC compared to the reference standard value of $1x10^7$ cfu g^{-1}. All producers exceed the reference standard TMC value by the analytical time T1 and increase again with further storage *(Figure 1)*. These findings are confirmed with even stronger evidence through the comparison at the different analytical times of each producer between the likelihood functions of the true log TMC, in the framework of the normal model (data not shown).

Regarding the EC levels, the representation proposed in *Figure 2* shows the distribution log EC in comparison to the threshold of 1000 cfu g^{-1}. Samples of Producer 3 exceed the reference standard value by the analytical time T1. The other two producers instead had values of EC within the limits during the entire shelf life *(Figure 2)*.

DISCUSSION

We are aware that to obtain a final RTE product of good quality, although the initial microbiological quality of the raw material is surely important, other factors such as post harvest handling, processing, storage and distribution could influence the levels of microbial contamination in RTE rocket salads at the point of sale.

This study describes the temporal pattern of the microbial quality of RTE rocket salad during its shelf life in Italy and its variation among three different procedures, representative of the Italian production systems of RTE salads. The most striking evidence is the high levels of TMC counts in all producers examined during the shelf life of the product. Although, at least for TMC, law limits are not exceeded, the safety of consumers is questioned as several pathogens might have similar dynamics to TMC.

Differences among producers are apparent and are possibly linked to the different minimal processing technologies followed by each of them after harvesting. For example, the control strategies based application of GAP in primary production, promotion of good hygienic practices, the strict application of the HACCP system during the processing, might be effective in reducing microbial contamination of rocket salad samples of Producer 1.

On the contrary, although Producer 2 disinfection with chlorinated water was effective in reducing bacterial contamination of the raw material (significant reduction of microbial charges between T0 and T1), TMC values were still higher than the values of the other two producers at T1 and exceed the reference standard value. Besides, the disinfection did not show the same effectiveness on EC *(Table 2)*. Finally, *Figure 1* and *Figure 2* give an immediate graphic evidence of the lower effectiveness of the Producer 3 handling procedures. Despite the good quality of raw material that showed low values of TMC, the rapid reduction of temperature of rocket salad used by Producer 3 before packaging was ineffective in reducing bacterial contamination, and is also well known that some pathogen microorganisms may continue to grow at low temperature even if at reduced growth rates [20, 21].

CONCLUSION

To obtain a RTE product of high hygienic quality is very important to start with a fresh raw material of high quality. The simple treatment (*e.g.* disinfection of Producer 2 and the rapid reduction of the temperature of Producer 3) applied to the raw rocket probably is not sufficient to guarantee the necessary hygienic quality, as also shown in other studies on RTE vegetables in Italy [22]. Washing fruits vegetables in potable water removes a portion of microbial cells and vigorous washing is suggested to be as effective as treatment with water containing 200 ppm chlorine, which generally reduces microbial populations by 10 to 100 fold [23]. However, the efficiency of removal of microorganisms greatly varies with the type of vegetable (*e.g.* depending on their surface characteristics), temperature and type of bacteria [21, 23].

Our study showed that of the 62 samples examined at the arrival time in commercial stores (24 h from packaging) 54.8% (34/62) and 21.0% (13/62) had levels of TMC and EC respectively above the reference limits. Clearly this results do not support an extension of the period of maximum shelf life of products analyzed.

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Conflict of interest statement

There are no potential conflicts of interest or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

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