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Growth of *Listeria monocytogenes* in Fresh-Cut Coconut as Affected by Storage Conditions and Inoculum Size

MILENA SINIGAGLIA,* ANTONIO BEVILACQUA, DANIELA CAMPANIELLO, DANIELA D'AMATO, AND MARIA ROSARIA CORBO

Department of Food Science, Agricultural Faculty, University of Foggia, via Napoli 25, 71100 Foggia, Italy

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ABSTRACT

In this study, the effects of packaging atmosphere (air and modified atmosphere, 65% N_2, 30% CO_2, and 5% O_2), temperature (2, 4, 8, and 12°C) and inoculum size (low inoculum, 10^2 CFU/g; high inoculum, 5×10^5 CFU/g) on the growth of *Listeria monocytogenes* in minimally processed coconut were investigated. Growth data were fitted to the Gompertz equation, and the sanitary risk time (the time, in days, necessary to observe an increase of 2 log CFU/g in the level of the pathogen) was also calculated. At a higher inoculum level, the health risk was more marked; moreover, as shown by parameter *A* of the Gompertz equation (maximum increase of cell load data), the sanitary risk time seemed not to be influenced by temperature or by atmosphere type. At a low inoculum level in the air-stored product, the sanitary risk time was strongly influenced by temperature, and a modified atmosphere caused a significant increase in the maximum cell load reached in the stationary phase. The results show that *L. monocytogenes* not only survives but is able to proliferate on fresh-cut coconut stored in air as well as in a modified atmosphere, even at a very low temperature (2°C); moreover, the presence of a high cell load can lead to a health risk because this pathogen can grow to risk level during the shelf life of the product.

Minimally processed refrigerated fruits have become a very important field for potential economic growth in the fresh-cut produce industry *(1)*. The shelf life of these products can be extended with high-quality raw products and careful control of temperature and relative humidity as well as by modified atmosphere packaging (MAP); however, fresh-cut fruits generally do not undergo any processing to eliminate pathogenic microorganisms before consumption. A long shelf life theoretically could provide time for pathogens to multiply without adversely affecting the organoleptic quality of the product and therefore could increase the risk of disease. *Listeria monocytogenes* is of special concern because it is able to grow both at refrigeration temperatures and under modified atmosphere conditions *(3, 6)*.

Coconut (*Cocos nucifera* L.) is one of the 10 most useful trees in the world. Humans can use every part of the coconut; the white nut-meat can be eaten raw or shredded and then dried and used in many cooking recipes. Fresh coconuts in the shell retain good quality for up to 1 month under refrigeration. Deshelled coconuts require protection from oxygen, which may cause the fat to become rancid; moreover, the white nut-meat is characterized by low acidity (about pH 6) and is susceptible to microbial spoilage *(17)*.

As with other fruits that are consumed raw, coconut may be a vehicle of pathogenic bacteria. In fact, although the coconut's shell protects against microorganisms, once this barrier is eliminated (e.g., by deshelling, slicing), the white meat can become contaminated and allow the growth of microorganisms. Refrigeration is used extensively to retard spoilage and extend the shelf life of fresh foods, including minimally processed fruits. To prevent listeriosis through the consumption of coconut, it is important to understand the characteristics of *L. monocytogenes* with respect to its ability to survive and grow in ready-to-eat (RTE) coconut. Studies related to *L. monocytogenes* growth in low-acid fruits are extremely limited. Penteado and Leitao *(14)* studied the behavior of this microorganism in melon, watermelon, and papaya and demonstrated that these low-acid fruits represent good substrates for the growth of *L. monocytogenes.*

In a previous study, Sinigaglia et al. *(17)* investigated coconut, as a raw material, for the production of RTE fruit. The objective of the present study was to investigate the effects of MAP and temperature on the growth of *L. monocytogenes* in coconut. Moreover, taking into account that previous studies *(5, 7, 10)* have demonstrated that bacterial growth is significantly dependent on initial cell density, the effect of inoculum size on *L. monocytogenes* growth during storage was evaluated.

MATERIALS AND METHODS

Bacterial strain and inoculum preparation. The strain of *L. monocytogenes* used in this study was from the "Collection of Zoo-prophylactic Institute of Apulia and Lucania Regions in Italy" and was isolated from cheese implicated in food poisoning in the Apulia region. The organism was transferred to fresh tryptic soy agar (Oxoid, Milan, Italy) periodically to maintain viability. The inoculum was prepared by two successive 24-h transfers of cells in tryptic soy broth (Oxoid) at 37°C, and the culture was centrifuged in an ALC 4239R centrifuge (ALC, Milan, Italy) at

* Author for correspondence. Tel: +39 0881 589233; Fax: +39 0881 740211; E-mail: m.sinigaglia@unifg.it.

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3,000 rpm (relative centrifugal force $(RCF)_{medium}$, 659 g; RCF_{max}, 995 g; RCF_{min}, 322 g; radius, 0.032 m) for 15 min at 4°C. The pellet was resuspended in sterile isotonic solution at a temperature of 4°C. To more precisely calculate the concentration of the inoculum, the optical density of the suspension was adjusted with isotonic solution in a spectrophotometer (600 nm).

The inoculation was carried out in the most homogeneous way possible, distributing the two inoculum concentrations of about 10^2 CFU/g (low inoculum) and 5×10^5 CFU/g (high inoculum) across the entire mass of coconut and then quickly and hermetically sealing the bag. The inocula were thoroughly distributed by externally hand massaging the bag for 1 min.

Fresh-cut coconut preparation and packaging. Fresh coconuts were purchased from a local supermarket, deshelled, and manually sliced with sharp knives. The inoculated slices (about 50 g of sample) were packaged in high-barrier plastic bags (nylon/polyethylene, 102 μm; Tecnovac, San Paolo D'Argon, Bergamo, Italy) by means of S100-Tecnovac equipment (Tecnovac). The bags were 170 by 250 mm long with the following manufacturer specifications: CO_2 and O_2 permeability, 3.26×10^{-19} mol m m^{-2} s^{-1} Pa^{-1} and 9.23×10^{-19} mol m m^{-2} s^{-1} Pa^{-1}, respectively; water vapor transmission rate, 1.62×10^{-10} kg m^{-2} s^{-1}. The samples were packaged in air and a modified atmosphere (65% N_2, 30% CO_2, and 5% O_2) and stored in Gallenkamp incubators (model 1R211GA, Sanyo/Gallenkamp, Bensenville, Ill.) at 2, 4, 8, and 12°C. The declared accuracy of the temperature control was ±0.2°C; moreover, external thermocouples were used to verify both the accuracy and uniformity of incubator temperature.

Fruit sampling and microbiological analyses. For microbiological analysis, coconut slices were cut into ca. 10-g pieces with sharp knives under sterile conditions. The weighed pieces of coconut meat were placed into a sufficient amount of 0.1% peptone solution to yield a 10-fold dilution and homogenized in a Sterilmixer II model 4153-50 (International PBI, Milan, Italy) for 120 s at room temperature. Serial dilutions of fruit homogenates were plated on selective medium and incubated. Specifically, direct counts of *L. monocytogenes* were performed by spreading 0.1 ml of the homogenized sample in peptone solution onto the surface of *Listeria* selective agar base (Oxoid CM856 plus *Listeria* selective supplement—Oxford formulation; Oxoid, SR140E), and the plates were incubated at 37°C for 48 h.

Microbiological data were expressed as the average of at least three replicates (three samples from three different packages). The variability coefficients, expressed as a percent ratio between the standard deviation and the mean value, were less than 7%.

The measurement of pH was performed on the first homogenized dilution of the coconut samples during storage with a Crison pH meter (model micro pH 2001, Crison, Barcelona, Spain).

Predictive modeling and risk assessment. The cell load data for *L. monocytogenes* that were collected during incubation of the samples were modeled according to the Gompertz equation as modified by Zwietering et al. *(19)*:

$$y = k + A \cdot \exp\{-\exp[(\mu_{max} \cdot e/A) \cdot (\lambda - t) + 1]\}$$

where y is the concentration of the microorganism (log CFU per gram), k is the initial cell count, A is the difference between the maximum population attained at the stationary phase and the initial count (log CFU per gram), μ_{max} is the maximal growth rate (Δlog CFU per gram per day), λ is the lag time (days), and t is the time.

The experimental data were statistically modeled through the Nonlinear Regression Procedure of Statistica for Windows (Statsoft, Tulsa, Okla.). The goodness of fit was evaluated on the basis of the regression coefficient (R).

The parameters of the Gompertz equation were analyzed by a one-way analysis of variance and Duncan's test ($P < 0.05$).

Following Castillejo Rodriguez et al. *(4),* the sanitary risk time for the growth of *L. monocytogenes* in our samples was determined from the time (in days) that it took to observe an increase of 2 log (CFU/g) of the count of this microorganism in foods as follows:

$$\text{sanitary risk time} = 2/\mu$$

where μ is the maximal growth rate.

The relationship between the sanitary risk time (y) and temperature was represented by the following linear equation:

$$y = a + b \cdot T$$

where a is the theoretical sanitary risk time at 0°C, T is the temperature (in degrees Celsius), and b (the slope) represents the increase in growth rate for each 1°C temperature increase. This parameter multiplied by a factor of 10 is the Q_{10} value.

RESULTS AND DISCUSSION

Fresh coconut is typically a low-acid fruit. Measured pH values averaged 6.0 pH units. These pH values cannot be considered inhibitory for *L. monocytogenes.*

Figure 1 shows the growth curves for *L. monocytogenes* inoculated with the high inoculum concentration in coconut slices packaged in air (Fig. 1A) and modified atmosphere (Fig. 1B) and stored at the various temperatures. It can be seen that the curves fit well with the experimental data and that, at every temperature, the microbial growth follows the classic pseudo-exponential trend described by the Gompertz function.

In Table 1, the estimated Gompertz parameters, as well as the R values, are reported. *L. monocytogenes* directly inoculated on fresh-cut slices of coconut was able to proliferate during storage at all tested temperatures, both in air and a modified atmosphere. A rise in storage temperature resulted in an increase in the growth rate and a decrease in the lag phase, both in air and a modified atmosphere. No significant differences ($P > 0.05$, Duncan's test) were observed between the air and the modified atmosphere.

Under all conditions, the initial population increased about 3 log CFU/g, reaching the stationary phase with a cell load ($k + A$) of 8 log CFU/g.

In a previous study of *L. monocytogenes* growth in fresh green asparagus, Castillejo Rodriguez et al. *(4)* reported that the sanitary risk time for *L. monocytogenes* could be calculated from the time (in days) it takes to observe an increase of 2 log (CFU per gram) in the count of this microorganism in foods, given that, under normal conditions, there is a very low level of this microorganism in foods *(8, 9, 12, 15).* Use of the growth rate (μ) of *L. monocytogenes* to calculate the sanitary risk time from the time (in days) it takes to observe an increase of 2 log (CFU per gram) in the count of *L. monocytogenes* is possible, because the growth rate is calculated from the slope of the exponential phase of the growth curve. This condition would represent the most unfavorable situation (that characterized by the fastest growth).

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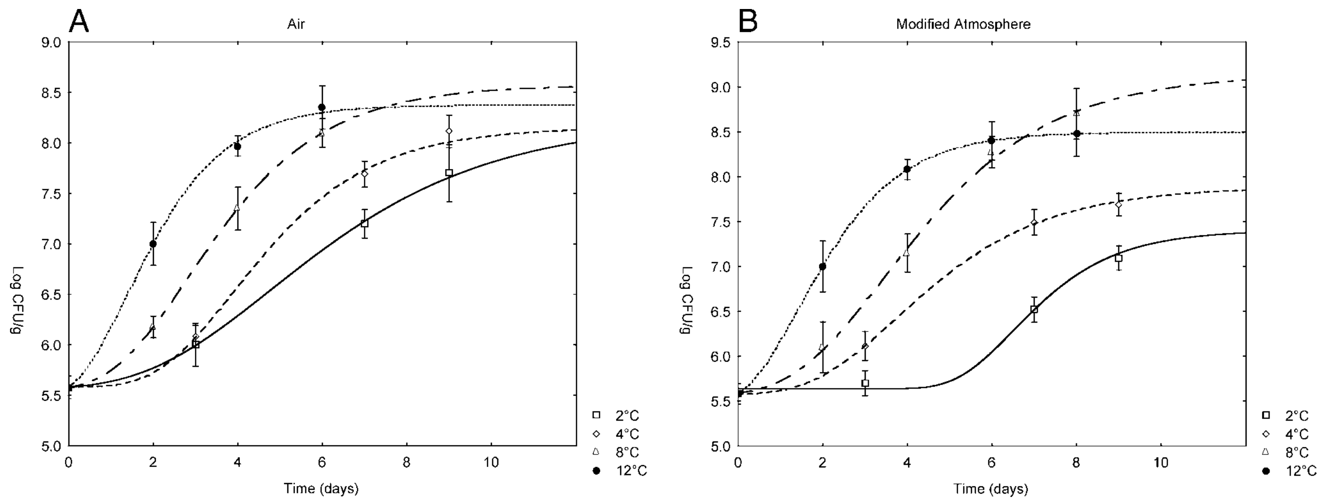

FIGURE 1. *Evolution of* Listeria monocytogenes *at a high inoculum level on slices of coconut packaged in air (A) and in a modified atmosphere (B) and stored at 2, 4, 8, and 12°C. Data are the average of three replicates and are accompanied by the standard deviation.*

In Table 1, this parameter is also reported. As can be observed, the storage of highly contaminated coconut at a temperature of 4°C could lead to a significant health risk, because *L. monocytogenes* is able to increase in number to a serious risk level in a very short time.

In air, the sanitary risk time varied from 2.32 to 6.06 days; in a modified atmosphere, the sanitary risk time ranged from 2.35 to 4.17 days. However, it was not possible to evaluate this index at 2°C, because the cell load did not increase by 2 log CFU/g during the experimental time (12 days).

When high initial numbers of *L. monocytogenes* are present in a food, the health risk is high, because even if growth does not occur, it is only necessary that the pathogens survive. In any case, typical experiments for predictive microbiology growth studies are usually conducted at high initial pathogen inoculum levels in food matrices so that almost all of the inoculated microorganisms can be recovered to avoid the necessity for preenrichment of the samples. However, high initial density is a factor that may lead to an overestimation bias. In fact, it has been shown *(5)* that density dependence is a factor in the growth rate of pathogens at suboptimal conditions, such as low temperature and acid pH. Moreover, Kaprelyants and Kell *(10)* suggested that the growth kinetics of bacterial populations are dependent on the initial population density as well as on the communication between cells, as demonstrated with eukaryotic cells. Therefore, the behavior of low concentrations of *L. monocytogenes* in minimally processed coconut was also evaluated. Figure 2 shows the growth of a low inoculum level of *L. monocytogenes* in coconut slices packaged in air (Fig. 2A) and a modified (Fig. 2B) atmosphere and stored at the various temperatures, as described in the experiments with the high inoculum levels. The Gompertz function described, very well, the trend of experimental data, as shown by the regression coefficients. The Gompertz parameters, as well as the *R* values, are reported in Table 2. *L. monocytogenes* inoculated at a low inoculum level on fresh-cut slices of coconut was able to proliferate during storage at the various temperatures, both in air and a modified atmosphere. An increase in storage temperature resulted in a significant reduction in the lag phase and an

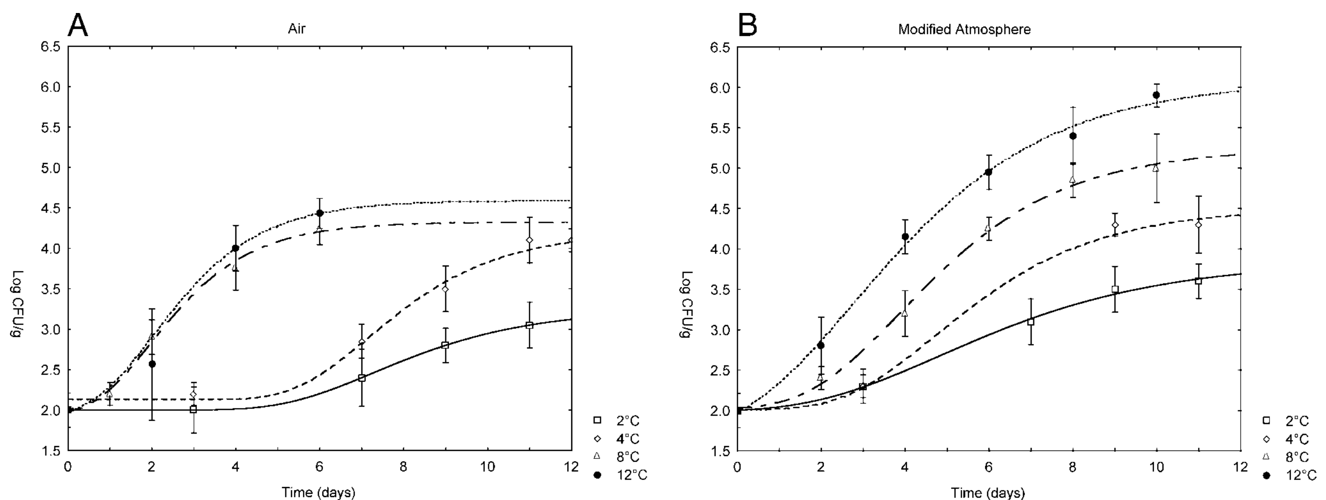

FIGURE 2. *Evolution of* Listeria monocytogenes *at a low inoculum level on slices of coconut packaged in air (A) and in a modified atmosphere (B) and stored at 2, 4, 8, and 12°C. Data are the average of three replicates and are accompanied by the standard deviation.*

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TABLE 1. *Gompertz parameters of* Listeria monocytogenes *at high inoculum levels on fresh-cut slices of coconut packaged in air and in a modified atmosphere and stored at 2, 4, 8, and 12°C*[a]

Temp (°C)[b]	*k*		*A*		μ_{max}		λ		*R*		Risk time[c]	
	Air	MA[d]	Air	MA	Air	MA	Air	MA	Air	MA	Air	MA
2	5.56 ± 0.06 a A[e]	5.63 ± 0.05 a A	2.66 ± 0.18 a A	1.76 ± 0.18 a B	0.33 ± 0.20 a A	0.42 ± 0.39 a A	1.82 ± 0.39 a A	5.16 ± 0.83 a B	0.999	0.998	6.06 b	—[f]
4	5.58 ± 0.13 a A	5.58 ± 0.06 a A	2.57 ± 0.21 a A	2.31 ± 0.09 b A	0.52 ± 0.10 b A	0.48 ± 0.04 a A	2.10 ± 0.48 a A	1.75 ± 0.07 b A	0.998	0.999	3.85 a A	4.17 a A
8	5.56 ± 0.08 a A	5.56 ± 0.11 a A	3.02 ± 0.19 b A	3.58 ± 0.32 c B	0.62 ± 0.07 c A	0.61 ± 0.06 b A	1.03 ± 0.32 b A	1.29 ± 0.39 b A	0.999	0.999	3.22 a A	3.28 a A
12	5.44 ± 0.14 a A	5.44 ± 0.01 a A	2.92 ± 0.06 b A	3.06 ± 0.01 d A	0.86 ± 0.10 d A	0.85 ± 0.01 c A	0.17 ± 0.03 c A	0.15 ± 0.02 c A	0.999	1.000	2.32 a A	2.35 a A

[a] Gompertz parameters: *k*, initial cell load (log CFU per gram); *A*, difference between the maximum cell load attained in the stationary phase and the initial cell count (Δlog CFU per gram); μ_{max}, maximal growth rate; λ, lag phase (days); *R*, regression coefficient. Data are accompanied by standard errors.

[b] Storage temperature.

[c] Risk time: time (days) necessary to have an increase of 2 log in the recount of *Listeria monocytogenes*. It is calculated by the maximal growth rate.

[d] MA, modified atmosphere (65% N_2, 30% CO_2, 5% O_2).

[e] The values in a column with the same lowercase letter are not significantly different ($P > 0.05$, Duncan's test). For each kinetic parameter, the values in a row with the same capital letter are not significantly different ($P > 0.05$, Duncan's test).

[f] —, not reached.

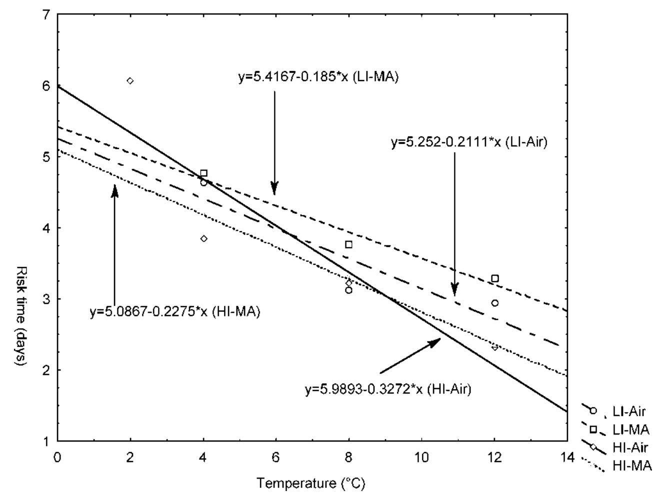

FIGURE 3. *Influence of storage temperature on the sanitary risk time of slices of coconut packaged in air and in a modified atmosphere at low and high inoculum levels of* Listeria monocytogenes.

increase in the growth rate, as shown by the results of Duncan's test. Moreover, the use of MAP caused a significant rise ($P < 0.05$) in parameter *A* for all of the experimental conditions. At 12°C, the maximum increase in the cell load was 2.63 log CFU/g in air and 4.35 log CFU/g in the MAP. However, at 2 and 4°C, populations of *L. monocytogenes* increased by 1.25 to 2.50 log in both atmospheres. These results confirm that low temperature is not a barrier to *Listeria* growth but that it can retard growth. The use of a modified atmosphere significantly influenced the duration of the lag phase, which was at a maximum in air at the lowest incubation temperatures (2 and 4°C).

In Table 2, the sanitary risk time, calculated according to Castillejo Rodriguez et al. *(4),* is also reported. The behavior of the pathogen did not seem to be influenced by the type of packaging atmosphere; in fact, the storage of coconut in both atmospheres at 4, 8, or 12°C led to similar health risks, with a sanitary risk time that ranged from 2.94 to 4.76 days. At 2°C, it was not possible to estimate the sanitary risk time, because the increase in cell load was lower than 2 log CFU/g.

These results are in agreement with previous reports *(2, 6),* demonstrating that MAP is ineffective at controlling *L. monocytogenes* growth. Moreover, it is important to note that the endogenous microflora of low-acid fruits and vegetables consist mainly of microorganisms capable of excreting cell wall–degrading enzymes, so they are able to gain access to the nutrients within the host cell to release nutrients *(11, 13).* Considering that these organisms are sensitive to CO_2, MAP can cause their suppression and, consequently, influence nutrient availability for other organisms, e.g., for some psychrotrophic pathogens, such as *L. monocytogenes.*

In a challenge study, it is very important to be aware of the role that naturally occurring microbiota play, because a high cell load of natural microflora could create an additional hurdle to the growth of the test microorganism. In coconut slices, the mesophilic cell load data attained in the

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TABLE 2. *Gompertz parameters of* Listeria monocytogenes *at low inoculum levels on fresh-cut slices of coconut packaged in air and in a modified atmosphere and stored at 2, 4, 8, and 12°C*[a]

Temp (°C)[b]	k		A		μ_{max}		λ		R		Risk time[c]	
	Air	MA[d]	Air	MA	Air	MA	Air	MA	Air	MA	Air	MA
2	2.00 ± 0.01 a A[e]	1.99 ± 0.09 a A	1.25 ± 0.01 a A	1.86 ± 0.27 a B	0.22 ± 0.07 a A	0.23 ± 0.01 a A	5.14 ± 0.01 a A	1.79 ± 0.83 a B	0.998	0.999	—[f]	—
4	2.13 ± 0.04 a A	2.01 ± 0.06 a A	2.08 ± 0.12 b A	2.50 ± 0.03 b B	0.43 ± 0.01 b A	0.42 ± 0.07 b A	5.38 ± 0.23 a A	2.54 ± 0.15 b B	0.997	0.993	4.63 a A	4.76 a A
8	1.94 ± 0.14 a A	2.02 ± 0.11 a A	2.38 ± 0.28 b A	3.22 ± 0.23 c B	0.64 ± 0.01 c A	0.53 ± 0.11 bc B	0.60 ± 0.39 b A	1.67 ± 0.43 a B	0.999	0.999	3.12 a A	3.77 a A
12	1.96 ± 0.17 a A	1.73 ± 0.68 a A	2.63 ± 0.29 c A	4.35 ± 0.68 d B	0.68 ± 0.08 c A	0.61 ± 0.09 c A	0.61 ± 0.23 b A	0.14 ± 0.10 c A	0.998	0.999	2.94 a A	3.28 a A

[a] Gompertz parameters: *k*, initial cell load (log CFU per gram); *A*, difference between the maximum cell load attained in the stationary phase and the initial cell count (Δlog CFU per gram); μ_{max}, maximal growth rate; λ, lag phase (days); *R*, regression coefficient. Data are accompanied by standard errors.
[b] Storage temperature.
[c] Risk time: time (days) necessary to have an increase of 2 log in the recount of *Listeria monocytogenes*. It is calculated by the maximal growth rate.
[d] MA, modified atmosphere (65% N_2, 30% CO_2, 5% O_2).
[e] The values in a column with the same lowercase letter are not significantly different ($P > 0.05$, Duncan's test). For each kinetic parameter, the values in a row with the same capital letter are not significantly different ($P > 0.05$, Duncan's test).
[f] —, not reached.

stationary phase varied from 6 to 8 log CFU/g (data not shown). The influence of the packaging atmosphere was not significant ($P > 0.05$), but a significant increase in cell counts ($P = 0.001$) was observed with an increase in temperature.

In Figure 3, the influence of storage temperature on sanitary risk time, according to microbial parameters relative to the growth of *L. monocytogenes,* is reported. The temperature dependence of fruit safety can be expressed by the Q_{10} value *(16),* which is defined as a 10-fold decrease in the sanitary risk time for a 10°C temperature increase. It can be observed that the sanitary risk time was affected very little by temperature when the pathogen inoculum level was low. Only in air with a high inoculum level was there a more evident influence of storage temperature, when the Q_{10} value was 3.27. This finding means that an increase in the storage temperature of 10°C decreased the sanitary risk time by 3.27 days.

At a low inoculum level, both in air and MAP, and at a high inoculum level in a modified atmosphere, the Q_{10} values varied from 1.85 to 2.28 days.

Comparing the sanitary risk times with the data of shelf life reported in a previous article *(17),* it can be observed that the storage of coconut slices at 4°C in both atmospheres could lead to significant risk, even when low levels of *L. monocytogenes* are present, because this pathogen is able to grow to risk level during the shelf life of the product. In fact, Sinigaglia et al. *(17)* reported that minimally processed coconut had a shelf life (calculated as the time to attain a total bacterial count value of 5×10^7 CFU g^{-1}) of about 7 days at 4°C.

These results confirm that coconut is a good substrate for *L. monocytogenes* growth. Thus, its presence in minimally processed coconut, whose intrinsic characteristics (e.g., pH, water activity) permit its growth, can be a risk factor. Moreover, the use of a modified atmosphere with a low amount of O_2 could cause a selective suppression of the growth of various epiphytic populations and promote the growth of the pathogen. Hence, the effect of competitive or synergistic microflora on the growth of pathogens under various gas-phase compositions must be evaluated.

This study has shown that *L. monocytogenes* not only survives but is also able to proliferate on fresh-cut coconut stored both in air and a modified atmosphere at a very low temperature (2°C). Although coconut may not be exposed to levels of *L. monocytogenes* as high as those used in this study, its capability to grow well on coconut slices at 2°C could represent a potential health hazard. Moreover, considering that the transfer of *L. monocytogenes* from the rind to the interior flesh during the preparation of fresh-cut fruits has been demonstrated *(18),* good hygienic practices and high standards of cleanliness when manipulating and storing this low-acid fruit are essential.

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