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Short communication

Modelling the growth of *Listeria monocytogenes* in fresh green coconut (*Cocos nucifera* L.) water

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ABSTRACT

The behaviour of *Listeria monocytogenes* in the fresh coconut water stored at 4 °C, 10 °C and 35 °C was studied. The coconut water was aseptically extracted from green coconuts (*Cocos nucifera* L.) and samples were inoculated in triplicate with a mixture of 5 strains of *L. monocytogenes* with a mean population of approximately 3 $\log_{10}$ CFU/mL. The kinetic parameters of the bacteria were estimated from the Baranyi model, and compared with predictions of the Pathogen Modelling Program so as to predict its behaviour in the beverage. The results demonstrated that fresh green coconut water was a beverage propitious for the survival and growth of *L. monocytogenes* and that refrigeration at 10 °C or 4 °C retarded, but did not inhibit, growth of this bacterium. Temperature abuse at 35 °C considerably reduced the lagtimes. The study shows that *L. monocytogenes* growth in fresh green coconut water is controlled for several days by storage at low temperature, mainly at 4 °C. Thus, for risk population this product should only be drunk directly from the coconut or despite the sensorial alterations should be consumed pasteurized.

1. Introduction

Coconut, *Cocos nucifera* L., which is a fruit commonly found in tropical regions of the world, presents a diversity of possible applications in several fields of food industries. Although coconut water had sometimes a status of subproduct by the industries, this beverage is traditionally appreciated and consumed by the habitants of coastal regions of tropical countries, as Brazil.

Coconut water is a drink with great concentration of mineral salts, besides having a pleasant flavour (Kwiatkowski et al., 2008) which have spread and increased its consumption. Coconut water was typically consumed direct from fruits, however currently there is a trend and search for aggregating practicality to the product. This is due to obstacles for consumers with respect to the transport, refrigerated storage and opening of the coconut husks. Thus, coconut water has been packed in plastic bottles and stored under refrigeration without a thermal process. Studies have showed that a thermal process causes a sensory change of the product and consumers have indicated a clear preference for the fresh beverage (Araújo et al., 2000; Frassetti et al., 2000).

Listeria monocytogenes is a psychrotrophic and ubiquitous pathogen (Gandhi and Chikindas, 2007). This pathogen presents a high mortality rate among some high risk groups, including the newborn, the aged and people with compromised immune systems (Slutsker and Schuchat, 1999). These people and pregnant women choose to consume coconut water due to its natural isotonic appeal and other claimed therapeutic properties. Although the incidence of *L. monocytogenes* has not yet been reported, other pathogens as *Salmonella* have been detected and counts reaching 10^5 CFU/mL of *Bacillus cereus* and *Staphylococcus aureus* in the fresh coconut waters maintained under refrigeration have been found (Melo et al., 2003; Leite et al., 1998; Hoffmann et al., 2002). Despite no listeriosis outbreaks associated with the consumption of coconut water have been found, vegetables have been recognized to harbour *L. monocytogenes* (Fröder et al., 2007; Little et al., 2007; Chen et al., 2009) or have been associated with listeriosis outbreaks (Schlech et al., 1983; Ho et al., 1986; Varma et al., 2007). The risk for coconut contamination by *L. monocytogenes* is increased due to the agricultural practices, since during harvest the fruits are placed directly on the soil, the natural habitat of *L. monocytogenes*. In addition, during fresh coconut water processing, there is no lethal step between extraction and packaging that assures beverage microbial safety. Furthermore, considering that the fresh beverage should be stored under refrigeration for up to one week, *L. monocytogenes* could find adequate conditions to growth and reach infective levels. Thus, the present study aimed at evaluating the survival/growth of *L. monocytogenes* in the fresh green coconut water, maintained at different

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common storage temperatures (4 °C, 10 °C and 35 °C). Predictive modelling was carried out by using Baranyi function in order to estimate growth kinetic parameters (growth rate, lag phase duration and maximum population).

2. Materials and methods

2.1. Coconut water extraction and assessment of sterility

The *in natura* coconut water was obtained from at least five green coconuts (*C. nucifera* L.) of the Dwarf variety, with seven months of maturation, the ideal harvest time (Aragão et al., 2002). The coconuts were acquired from the Campinas Supply Centre, State of São Paulo, Brazil. The fruits were manually washed with a sponge and alkaline detergent, and the surface then flamed so as to promote external sterilisation. In a bio-safe chamber of the laminar flow type, the fruits were cut parallel to the calyx using a sterile knife, so as to expose the mesocarp, and then perforated with a cylindrical borer. The coconut water from each fruit was aseptically extracted with the aid of a sterile dispenser and transferred to separate sterile flasks. The sterility of the coconut waters was tested before the *L. monocytogenes* predictive modelling studies. 5 mL aliquots of coconut water from the stock flasks were aseptically transferred to flasks containing 45 mL of trypticase soy broth supplemented with 0.6% yeast extract (TSB-YE) and incubated at 35 °C for 10 days. During this period, the coconut waters were stored at −18 °C. If the samples inoculated in TSB-YE showed no sign of turbidity, the coconut waters were considered to be sterile, and they were then defrosted and mixed for use in the experimental trials.

2.2. Chemical and physicochemical characterisation of the coconut water

The coconut water was submitted to the following chemical and physicochemical analyses: titratable acidity and reducing and total sugars (Adolfo Lutz Institute, 1985), determination of the pH using a Digimed model DM-20 pH metre (Digicron Analítica Ltda., São Paulo, Brazil), a_w in an Aqualab model 3TE water activity analyser (Decagon Device Inc., Pullman, USA) and of soluble solids using an AbbeÂ Carl Zeiss model 32-G 110d refractometer (Jena, Germany). All these determinations were carried out in triplicate.

2.3. Bacterial cultures and preparation of inoculum

The inoculum was composed of 5 strains of *L. monocytogenes*: ATCC 19111 (serovar 1/2a, isolated from chicken), IOC 1359 (serovar 4b, isolated from cheese), IOC 1527 (serovar 1/2a, isolated from frozen cooked meat), IOC 1898 (serovar 1/2a, isolated from spinach) and Scott A (serovar 4b, clinical isolate). The strains did not show any cross-strain inhibition, as revealed by the test described by Beuchat et al. (2001). The test revealed no cross-strain inhibition among the strains investigated. Further, strains were confirmed by biochemical tests (Pagotto et al., 2001). The cultures were maintained at 4 °C in TSA-YE until used.

The 5 strains of *L. monocytogenes* were cultivated individually in 10 mL TSB-YE at 35 °C, and the cultures transferred at three successive 24 h intervals. The cell suspensions were centrifuged (2000 × *g* for 15 min, 4 °C), the supernatants discarded and the cell sediments washed with 10 mL 0.1% peptone water. The centrifugal procedure was repeated and the cell masses re-suspended in 10 mL 0.1% peptone water. Aliquots of the same volume of the 5 suspensions were combined and the concentration of the mixture adjusted to unit 2 of the McFarland standard scale in a Densimat (bioMérieux SA, Marcy-l'Etoile, France) using a saline solution (0.85% NaCl). Preparation of the inoculum with approximately 10^5 CFU/mL was finalised by serial decimal dilution of the mixture in 0.1% peptone water, and immediate inoculation into the coconut waters. The viable cell concentrations in the 5 individual suspensions and in the mixture were determined by spread plating count on TSA-YE, with incubation at 35 °C for 24h.

2.4. Inoculation of coconut water and monitoring procedures

The coconut water samples (99 mL) were previously maintained in water baths at the studied storage temperatures (4, 10 and 35 °C) for 2 h in order to assure the adequate experimental temperature before *L. monocytogenes* inoculation. The coconut water samples were each inoculated with 1 mL of the *L. monocytogenes* suspension, to reach a level of 10^3 CFU/mL. This inoculum level was chosen based on Institute of Food Technologists (2003), which indicates that a level between 10^2 and 10^3 CFU/mL is typically used in microbiological challenge tests. After the inoculation, the flasks were shaken for 10 s.

For *L. monocytogenes* growth monitoring, aliquots of coconut water serial diluted in 0.1% peptone water were inoculated onto the surface of TSA-YE, following incubation at 35 °C/24 h and counting. The coconut waters incubated at 4, 10 and 35 °C were analysed at successive intervals of at least 6 days, 24 h and 6 h, respectively. Previous experiments (data not shown) have demonstrated that these time intervals were required to adequately adjust the data points to the models used. Three to five isolated colonies at each analysis points were subjected to biochemical confirmation (Pagotto et al., 2001). Negative controls, samples not inoculated with *L. monocytogenes*, were also incubated at the experimental temperatures, so as to assure the absence of the contaminant in the coconut waters during the periods studied.

2.5. Predictive modelling

The Baranyi's DMFit Excel add-in software (www.ifr.ac.uk/safety/DMfit) was used to fit the experimental growth data to the Baranyi model equation (Baranyi and Roberts, 1995) (equations (1)–(3)).

$$y(t) = y_0 + \mu_{\max} A(t) - \frac{1}{m}\left[1 + \frac{e^{m\mu_{\max}A(t)} - 1}{e^{m(y_{\max} - y_0)}}\right] \quad (1)$$

$$A(t) = t + \frac{1}{\mu_{\max}} \ln\left(\frac{e^{(-\mu_{\max}t)} + q_0}{1 + q_0}\right) \quad (2)$$

$$\lambda = \frac{\ln\left(1 + \frac{1}{q_0}\right)}{\mu_{\max}} \quad (3)$$

where $y(t)$ is the $\log_{10}$ of the cell concentration, at time t [h]; and $y_{\max}$ (κ) is the $\log_{10}$ of the maximum cell concentration; y_0 is the $\log_{10}$ of the initial cell concentration, $\mu_{\max}$ [1/h] the maximal specific growth rate; q_0 is a measure of the physiological state of cell when $t = t_0$; m is the parameter related to curvature after exponential phase.

The growth kinetic parameters determined by using Barany model were: λ – lagtime (hour), $\mu_{\max}$ – maximum growth rate, {[$\log_{10}$(CFU/mL)]/h} and κ – maximum population ($\log_{10}$ CFU/mL).

Data obtained were compared with those predicted by Pathogen Modelling Program (PMP, version 7.0) from the US Department of Agriculture-Agricultural Research Service (USDA-ARS). PMP set for *L. monocytogenes* growth in BHI broth was in the pH and water activity values determined for fresh green coconut water, as presented in Table 1. The initial *L. monocytogenes* count was set in the same level inoculated in fresh green coconut water (3 $\log_{10}$ CFU/mL).

2.6. Data analyses

For each temperature studied, triplicate growth curves were performed. For each growth curve point, duplicate plates were used to enumerate *L. monocytogenes* in fresh green coconut water. *L. monocytogenes* counts were transformed to $\log_{10}$ CFU/mL and graphs were drawn using Excel. Descriptive statistical calculations were also applied to the data of each growth kinetic parameter to determine the mean and standard deviation. Data were checked for significant statistical ($P \leq 0.05$) differences through Analysis of Variance and Tukey test using the Statistica 7.0 for Windows programme. The model was evaluated by calculating the coefficient of determination (R^2).

3. Results and discussion

This is the first report on the survival and growth of *L. monocytogenes* in fresh green coconut water. Here we have also shown primary predicted growth parameters in a broad range of temperatures to which the product could be exposed after its extraction, during its commercialization and consumption. This study was based on the trend and need to know the microbial ecology of fresh fruit juices or beverages better, which are highly appreciated by a variety of consumers world-wide.

Fresh green coconut water sterility tests indicated that contamination was absent in the samples extracted and consequently in the product inside the fruit. The samples extracted were stored as controls for selected monitoring periods (73 days at 4 °C, 15 days at 10 °C and 2 days at 35 °C, respectively). The results found here showed that the procedure adopted to extract the fresh coconut water was efficient in avoiding microbial contamination. The confirmation of 3–5 colonies amongst those counted during the *L. monocytogenes* growth trials (initial, middle and final phases of the curves), also showed that only this microorganism grew in the fresh green coconut water, guaranteeing confidence in both the procedure applied and the data obtained.

The physicochemical characteristics of the fresh green coconut water used (Table 1) are representative of what is reported in the literature (Jayalekshmy et al., 1986; Kwiatkowski et al., 2008). The water activity and pH values found classify the fresh green coconut water as a low acid and high water activity beverage. The sugar contents and other soluble solids provide carbohydrates for microbial growth and together with the previous characteristics make this beverage highly prone to the survival and growth of pathogens.

Fig. 1 shows that *L. monocytogenes* could grow in the fresh green coconut water at all the temperatures evaluated (4 °C, 10 °C and 35 °C). Considering that this beverage is not submitted to any inactivation step before consumption, that *L. monocytogenes* is a ubiquitous microorganism (Gandhi and Chikindas, 2007) and that the beverage is stored under refrigeration temperatures during its commercialization, this pathogen should be considered as a potential challenge for the safety of fresh green coconut water. This should be reinforced considering the reports of pathogens associated with fruits and fresh fruit juices/beverages and their association with disease caused by fruit juices (Vojadni et al., 2008). The ability of *L. monocytogenes* to grow in low acid fruits/fruit pulps, such as melon, watermelon, papaya (Penteado and Leitão, 2004), persimmon (Uchima et al., 2008) and in fresh green coconut water, as shown here, has been reported. However, the reasons for the non-association between this pathogen and fruit/juice disease outbreaks are not clear at this moment, whereas such an association is clear for *E. coli* 157:H7 and *Salmonella* spp in apple cider and/or non-pasteurized orange juice (CDC, 1996, 1999).

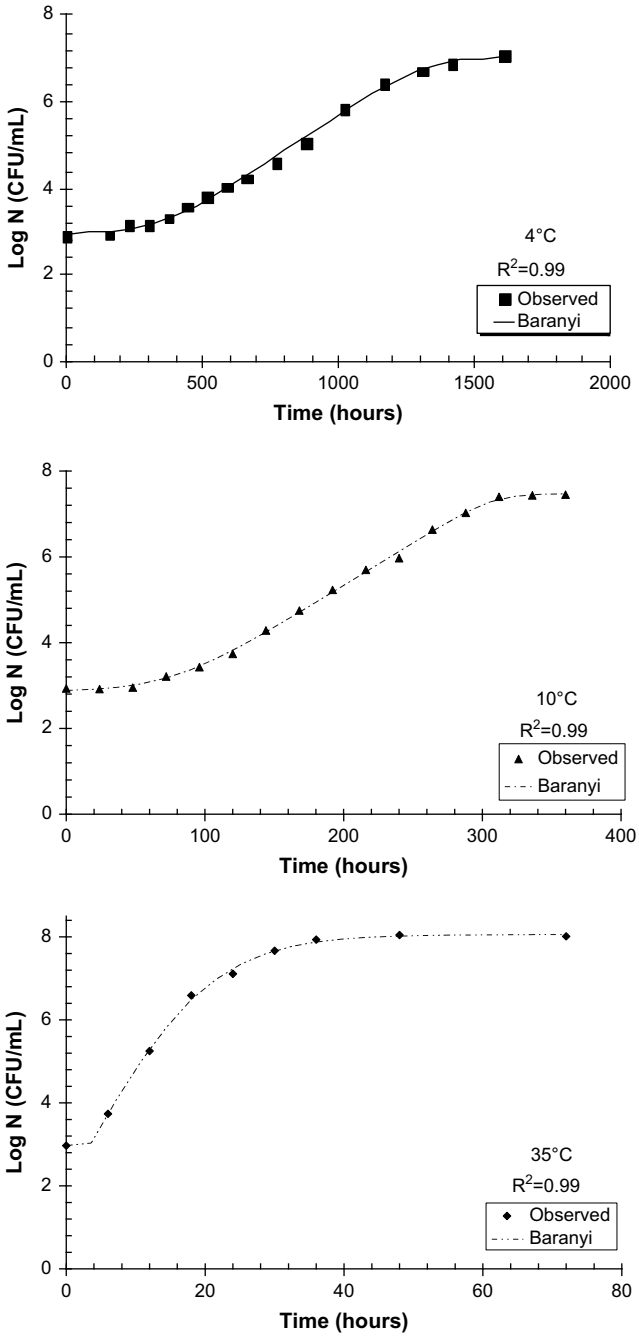

Fig. 1. Growth of *L. monocytogenes* in green coconut water stored at 4 °C, 10 °C and 35 °C. Points represent a mean of triplicate results.

Table 1
Physicochemical characteristics of coconut water used in the *L. monocytogenes* growth trials.

Physicochemical parameters	Values[a]
pH	4.88 ± 0.05
Water activity	0.995 ± 0.003
Titratable acidity (%)	1.12 ± 0.08
Solid soluble (°Brix)	5.47 ± 0.06
Total sugars (%)	3.98 ± 0.02
Reducing sugars (%)	3.97 ± 0.04

[a] Mean ± SD ($n = 3$).

Table 2
The growth kinetic parameters of *L. monocytogenes* in fresh green coconut water stored at different temperatures and comparison with prediction given by the Pathogen Modelling Program.[a,b]

Storage temperature (°C)	Growth rate ([$\log_{10}$ CFU/mL]/h)		Lagtime (h)		Maximum population ($\log_{10}$ CFU/mL)	
	Fitting to the Baranyi Model	PMP Prediction[c]	Fitting to the Baranyi Model	PMP Prediction[c]	Fitting to the Baranyi Model	PMP Prediction[c]
4	0.0043 ± 0.00044[c]	0.0059 [0.0052–0.0068]	357.16 ± 184.62[a]	353.3 [291.4–428.5]	7.09 ± 0.03[c]	9.47 [9.34–9.53]
10	0.0198 ± 0.0017[b]	0.0191 [0.0172–0.0210]	76.70 ± 23.83[b]	128.8 [112.2–147.8]	7.47 ± 0.24[b]	9.55 [9.52–9.56]
35	0.3044 ± 0.0390[a]	0.3010 [0.2508–0.3762]	3.50 ± 0.60[c]	8.5 [6.8–10.6]	8.16 ± 0.06[a]	9.46 [9.28–9.54]

[a] Different lower-case letters for values in the same columns indicate significant statistically differences ($P \leq 0.05$).
[b] Standard deviations were determined from kinetic parameters estimated for each one of the three growth curves.
[c] PMP provides 95% confidence intervals. PMP prediction for pH = 4.88 and $a_w = 0.995$.

Graphs presented in Fig. 1 were fitted by Baranyi model. Determination coefficients (R^2) values for storage temperatures were 0.99 indicating a very good fitness of model to the data. Table 2 shows predicted growth parameters for *L. monocytogenes* in fresh green coconut water stored at 4 °C, 10 °C and 35 °C.

The reduction of the storage temperature of the fresh green coconut water from 35 °C to 10 °C and then to 4 °C leads to a reduction in *L. monocytogenes* growth rate by approximately 15 and 70 times, respectively. Significant differences for this parameter ($P < 0.05$) were observed for all temperatures studied. PMP predicted very close values for μ_{max}, showing that the growth of *L. monocytogenes* in fresh green coconut water and BHI (used for PMP predictions) was similar.

An extended lagtime (λ) is the major focus of the non-lethal preservative methods applied to foods. Values of lagtimes were also significantly different ($P < 0.05$) for all temperatures assessed. The reduction of storage temperature from 35 °C to 4 °C, implied in approximately 100 times increasing in lagtimes, showing that storage temperature is an effective way for controlling *L. monocytogenes* in fresh green coconut water. It is interesting to note that the standard deviation for the lagtime under the most stressful conditions (4 °C) was higher than that for the most favourable one (35 °C). The values for the standard deviation reached up to 50% of the mean value for the lagtime at 4 °C, while they were reduced to 25% and 15% of the mean values when the temperature conditions for the growth of *L. monocytogenes* were 10 °C and 35 °C, respectively. These findings could be a result of the high variability found between the cells which are more evident when the conditions to which the microorganism is subjected are stressful. The variability in the standard deviations for the lagtimes of up to 60% of the mean value has also been shown in a recent study at the lower temperature studied here (10 °C), while for the higher temperatures of 20 °C and 30 °C, the standard deviations reached approximately 30% of the mean values (Uchima et al., 2008). Regarding PMP predictions, it was observed that while values found for κ at 4 °C were close to those predicted by Baranyi model, at 10 and 35 °C, they were approximately the double.

The κ values for *L. monocytogenes* in fresh green coconut water were significantly different for all conditions studied. The higher the storage temperature the higher the κ values ($P < 0.05$). *In natura* coconut water producers generally establish a shelf life of up to 1 week for the packed beverage when maintained under refrigeration. During this period, the population of *L. monocytogenes* in the coconut water stored at 4 °C remained practically unaltered, whereas that stored at 10 °C increased by almost 1 $\log_{10}$ cycle, after a latency period of approximately 3.5 days. At 35 °C the bacterium presented a rapid generation time ($\sim$1 h), and thus temperature abuse or the difficult to maintain the product under 4 °C during 1 week storage can considerably increase the level of potential hazards in coconut water.

Maximum population (κ) presented discrepancies when compared with predictions by Baranyi model, and overall, PMP predictions yielded higher values for this parameter particularly at 4 and 35 °C. This is explained by the fact that the Gompertz model (used in PMP) tends to overestimate the maximum population density, particularly when the number of data points during the stationary phase is limited (Buchanan et al., 1997). In this model, the parameter estimated is the maximum rate and that the maximum rate always occurs at an arbitrary point of inflection, the downward projection of this maximum rate to an arbitrary asymptote, not always matching the initial concentration, defines the end of the lag period (Garthright, 1991, 1997). In addition, the discrepancies in κ values obtained could be result of differences in nutrients contents between fresh green coconut water and BHI (used for PMP predictions).

The characteristics of coconut water associated with the production and storage conditions, the ability of *L. monocytogenes* to grow in the product and also the increase growth in the product consumption can make this fresh beverage a potentially hazardous food. This emphasises the importance of good agricultural and manufacturing practices when obtaining and packaging this beverage.

Although the implantation of good practices can serve to reduce contamination of the coconut water it cannot eliminate the possibility of the presence of pathogens such as *L. monocytogenes*. Then, in addition to hygiene controls during coconut water extraction and processing, consumer education and temperature control during commercialization and consumer phases are other special controls in order to assure fresh green coconut water safety. The consumers should also be informed about the correct manipulation of the beverage. Pregnant women, newborn, aged and people with compromised immune systems should only consume the coconut water obtained direct from fruit. Even with sensorial alterations, the consumption of the pasteurized coconut water would be an alternative.

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