Enterococcus faecalis and *Pseudomonas aeruginosa* behaviour in frozen watercress (*Nasturtium officinale*) submitted to temperature abuses

Sílvia R. Oliveira[a], *Rui M.S. Cruz*[b], *Margarida C. Vieira*[a], *Cristina L.M. Silva*[b], *Maria N. Gaspar*[a,*]

[a]*Laboratório de Microbiologia, Escola Superior de Tecnologia, Universidade do Algarve, Campus da Penha, 8005-139 Faro, Portugal*
[b]*Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal*

Keywords:
Freezing
Thawing
Salad
Watercress
Cultivar
Survival
Enterococcus
Pseudomonas

ABSTRACT

Watercress is an herb traditionally consumed fresh. If frozen, would be readily available to consumers. However, pathogenics resistant to frozen storage are a safety concern in this new product. In this study watercress was artificially contaminated with *Pseudomonas aeruginosa* ATCC 27853 and *Enterococcus faecalis* ATCC 29212. Their survival was evaluated after blanching, frozen storage and temperature fluctuations of the frozen product. The blanching caused a reduction of about 2 log cfu per gram of product of total viable count (TVC) and about 1.7 and 1.3 log cfu per gram of product of *P. aeruginosa* and *E. faecalis*, respectively. *P. aeruginosa* seemed to be more sensitive to temperature abuses than *E. faecalis*. After 3 months, TVC was still observed with a reduction of about 3 log cfu per gram of product. At the end of the study, exposure to freeze–thaw cycles resulted in death or injury of the microorganisms. These findings on the behaviour of two microorganisms of concern in frozen watercress will help improving the safety and cold chain settings for this product.

Enterococcus faecalis et Pseudomonas aeruginosa : comportement dans du cresson (*Nasturtium officinale*) congelé assujetti à des hausses de température

Mots clés : Congélation ; Décongélation ; Salade ; Cresson ; Variété ; Survie ; *Enterococcus* ; *Pseudomonas*

* *Corresponding author*. Tel.: +351 289 800151; fax: +351 289 888405.
E-mail addresses: silviapdoliveira@gmail.com (S.R. Oliveira), rcruz@ualg.pt (R.M.S. Cruz), mvieira@ualg.pt (M.C. Vieira), clsilva@esb.ucp.pt (C.L.M. Silva), mngaspar@ualg.pt (M.N. Gaspar).

Introduction

Preservation techniques, such as drying, salting, heating or freezing, rely on the inactivation/inhibition of spoilage/pathogenic microorganisms. Freezing has been used, since ancient times, as a physical process to extend products shelf-life (Geiges, 1996). Frozen vegetables are often subjected to a pre-process, such as blanching, and are ''quick-frozen'' in order to maximize their quality attributes. The blanching process is a mild heat treatment with an important role on the inactivation of enzymes, the reduction of microorganisms and colour stabilisation (Arthey, 1995). On the other hand, the ''quick freezing'' process assures that the maximum ice crystallization zone is passed through as quickly as possible (Lund, 2000), thus minimizing the negative impact on quality.

Blanching time/temperature conditions reduce, to varying extents, the number of viable microorganisms (Archer, 2004). Each and every food product harbours its own specific and characteristic microflora, which is a function of the raw material flora, processing, preservation and storage conditions. Based on the knowledge of a few chemical and physical parameters, it is also possible with great accuracy to predict which microorganisms may grow and dominate in a particular product (Gram et al., 2002).

The freezing process is generally an excellent way to control microbial growth. On the other hand, repeated freeze–thaw cycles disrupt and destroy bacteria. Effects of cyclic freezing on most microbial pathogens are not well documented (Archer, 2004). As stated by Lund (2000), Gram-negative bacteria are more susceptible to freezing than Gram-positives, but some of the Gram-negative microorganisms may survive well in frozen foods, depending essentially on the nature of the food matrix. The vegetative cells of micrococci, staphylococci and streptococci, in particular *Enterococcus faecalis*, are very resistant to freezing and frozen storage conditions (Geiges, 1996). The Gram-negative food-borne pathogens cause the vast majority of food-borne illness (Archer, 2004), therefore understanding their behaviour in frozen foods is important. Food products of vegetable origin present a special case, due to their nutrient composition. The relatively high pH value will allow a wide range of Gram-negative bacteria to grow, and spoilage is specially caused by organisms capable of degrading the vegetables polymer, pectin (Liao, 1989; Liao et al., 1997). These organisms, typically *Erwinia* and *Pseudomonas* species, have been recognised as spoilage organisms of several ready-to-eat vegetable products (Nguyen-The and Prunier, 1989; Lund, 1992).

In the commercial chain of the deep-frozen foods sale, single rises in temperature can occur when products are being sequentially transferred to a lorry, to a truck, to the storage room of the retail store, and from the time when consumers take a product from the freezer unit to the time when the product reaches the household freezer (Geiges, 1996). These temperature abuses (fluctuating temperatures) must be avoided, since they might have a negative influence on the food product safety, and even more on its nutritional and sensorial quality.

Watercress (*Nasturtium officinale*) is an herb found in and around water, normally consumed fresh or cooked in various kinds of recipes. Under refrigerated storage it has a short shelf-life of approximately seven days, however, it could be readily available to consumers if frozen. Pathogenic contaminants resisting frozen storage are a safety concern in this new product.

The objective of this study was to understand the survival of *Pseudomonas aeruginosa* ATCC 27853 and *E. faecalis* ATCC 29212 in frozen watercress when submitted to temperature abuses, according to a pre-established plan based on a real situation, as compared to maintenance at −21 °C.

Materials and methods

Baccterial strains and preparation of inoculum

The inoculum used in this study was a mixture of two strains from the American Type Culture Cells: *E. faecalis* ATCC 29212 and *P. aeruginosa* ATCC 27853. The microorganisms were grown on Plate Count Agar (PCA) medium (Merck, Darmstadt, Germany) at 37 °C. One loop full of each microorganism was transferred to 250 ml of Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke, London) and incubated at 37 °C for 16 h, until the population levels were 10^7 cfu ml^{-1}. The inoculum used, to artificially contaminate watercress samples, contained a final population level of 10^5 cfu ml^{-1}.

Watercress inoculation, blanching and freezing

Watercress was kindly supplied by Vitacress Company (Algarve-Portugal). Two types of samples were studied, contaminated and non-contaminated. To prepare the contaminated samples, approximately 120 g of watercress leaves were carefully selected and introduced in the contaminated broth for 10 min. Both contaminated and non-contaminated leaves were passed through a sterilized colander, washed with sterilized water and blanched at 95 °C in water during 20 s (Cruz et al., 2006). The watercress leaves were compressed in a high-density polyethylene mould into a slab shape (4.6 × 3.3 × 1.8 cm). Slab duplicates were packed in low-density polyethylene bags (14 × 25 cm) and frozen in an air blast freezer at −25 °C and 8 m s^{-1} (Armfield FT 36, Hampshire, England).

Storage and sampling

Part of the frozen contaminated watercress slabs were submitted to temperature abuses, according to a pre-established plan, and microbiological analyses were performed in each step marked in Fig. 1 (*S1–S5* correspond to production and distribution; *S6–S7* correspond to consumer product purchase and storage). Non-abused contaminated watercress was also studied, and in this case frozen slabs were maintained at −21 °C (Haier HF-248, Germany) during 3 months and analysed every month. Non-contaminated watercress was incubated under the same conditions of the contaminated one, with and without temperature abuses.

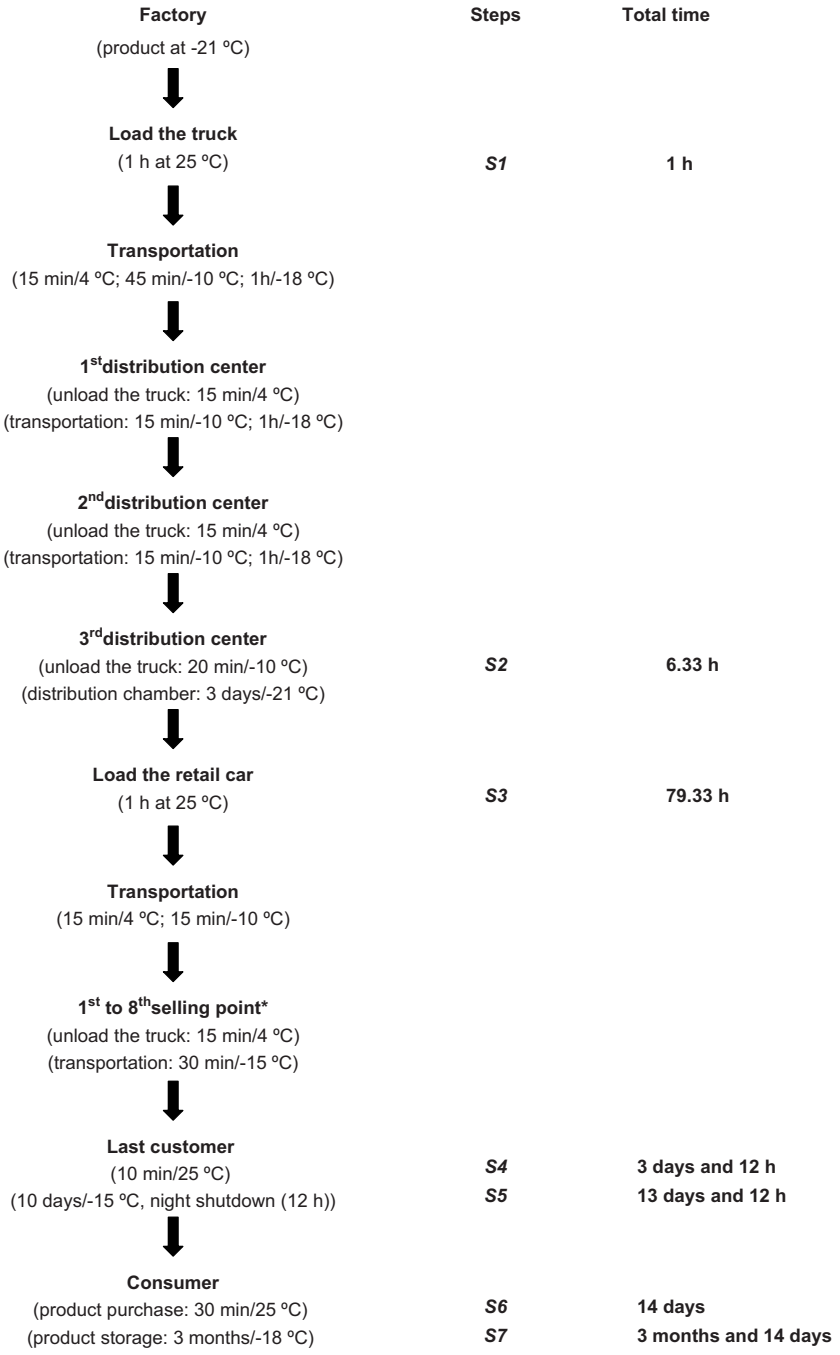

Fig. 1 – Pre-established plan of temperature abuses, based on a real situation, applied to the frozen watercress packages, simulating distribution and storage conditions over a period of three months: (S1–S7) corresponding to microbiological analyses; * These two steps were repeated 8 times.

Microbiological analyses

Microbial counts were performed in fresh, blanched and frozen watercress prior to the study, and in non-contaminated and artificially contaminated samples, with and without temperature abuses. The incidence of naturally occurring *P. aeruginosa* and *E. faecalis* strains on the fresh product was also evaluated.

For microbiological analyses, 10 g of each watercress sample were homogenised in 90 ml of Buffered Peptone Water (BPW) (Oxoid) in a Stomacher bag (Seward, Stomacher 400), using a blender Stomacher (Seward, Stomacher 400, London, England) for 120 s. Appropriate decimal dilutions were prepared from the homogenate with BPW.

Total viable counts (TVC) were obtained by incorporating inoculum of various dilutions on PCA Agar. Colonies were counted after 48 h of incubation at 30 °C (NP-1409/1987).

To determine numbers of *E. faecalis* ATCC 29212 (Roberts et al., 1995), spread plates on Slanetz and Bartley Glucose Azide medium (Oxoid) were incubated initially at 37 °C for 4 h,

to allow resuscitation of stressed cells, and then at 44 °C for 44 h. Maroon, pink and red colonies were counted and tested to confirm identity, using Kanamicin Aesculin Agar (Oxoid), BHI broth with 6.5% NaCl (Oxoid), catalase test and Gram stain.

P. aeruginosa ATCC 27853 counts on the product were performed based on the method reported by Roberts et al. (1995), by spread plate on Pseudomonas Agar base (Merck), containing glycerol (10 ml l^{-1}) (Merck), selective agents cetrimide (200 mg l^{-1}) (Oxoid) and sodium nalidixate (15 mg l^{-1}) (Oxoid), incubated at 37 °C for 7 days, before counting. Confirmation tests of the colonies were performed on Pseudomonas Agar F base (Merck) under U.V. light (Lamag-Cabinet II, Wiehl, Switzerland). Oxidase test (Merck) and Gram stain were also performed. The experiments were run in duplicates.

Statistical analysis

A single-factor analysis of variance (ANOVA) was carried out, for the studied microorganisms, to test any significant differences between each step (Excel-Microsoft Corporation, 2007). Evaluations were based on a significance level of 5%.

Results

The results confirmed that *P. aeruginosa* is a natural occurring microorganism in watercress, with counts of about 3.8 log cfu per gram of product (Table 1).

The blanching process of artificially contaminated watercress significantly reduced ($P < 0.05$) numbers of TVC, with a decrease of about 2 log cfu per gram of product. The microbial counts of *P. aeruginosa* ATCC 27853 and *E. faecalis* ATCC 29212 were also significantly reduced ($P < 0.05$) by about 1.7 log cfu per gram of product and 1.3 log cfu per gram of product, respectively. The freezing process (Table 1) slightly reduced the microbial counts of all the samples.

In artificially contaminated frozen watercress submitted to temperature abuses (Fig. 1-*S7*) the reduction of total viable counts was significant ($P < 0.05$), about 3 log cfu per gram of product. No survival of *P. aeruginosa* ATCC 27853 and *E. faecalis* ATCC 29212 was observed after 10 days at −15 °C (Fig. 1-*S5*) and when left 30 min at 25 °C (Fig. 1-*S6*), respectively (Fig. 2a).

The reduction ($P < 0.05$) of TVC during frozen storage of artificially contaminated watercress without temperature abuses was about 2 log cfu per gram of product. No survival of *P. aeruginosa* ATCC 27853 was observed after 2 months at −21 °C, while for *E. faecalis* ATCC 29212 the same was observed only after 3 months at the same temperature (Fig. 2b).

The corresponding results obtained for non-contaminated frozen watercress are presented in Fig. 3. *E. faecalis* was not detected in the fresh watercress. In the case of *P. aeruginosa* although the counts in the fresh product were about 3.8 log cfu per gram, after the freezing process no colonies were observed (Table 1). A reduction of around 3 log cfu per gram of product was observed in TVC (Fig. 3a) since the beginning till the end of the temperature abuses plan (Fig. 1).

Table 1 – Numbers of total viable counts (TVC 30 °C), *Pseudomonas aeruginosa* ATCC 27853 and *Enterococcus faecalis* ATCC 29212 in watercress samples

	TVC 30 °C	*P. aeruginosa* ATCC 27853[a]	*E. faecalis* ATCC 29212[a]
Fresh	7.09 ± 0.63	3.81 ± 0.41	nd
Contamination	7.60 ± 0.22[c]	7.04 ± 1.10	7.31 ± 0.51
Washing	7.29 ± 0.02[c]	6.66 ± 1.03	6.23 ± 1.56
Blanching	4.91 ± 0.22[b]	0.97 ± 1.33	nd
	5.29 ± 0.00[c]	4.97 ± 0.83	4.97 ± 0.53
Freezing	4.51 ± 0.01[b]	nd	nd
	4.59 ± 0.05[c]	4.54 ± 0.33	4.51 ± 0.15

± = Standard deviation; nd = not detected.
a Results expressed as log 10 cfu per gram of product.
b Non-contaminated watercress.
c Artificially contaminated watercress.

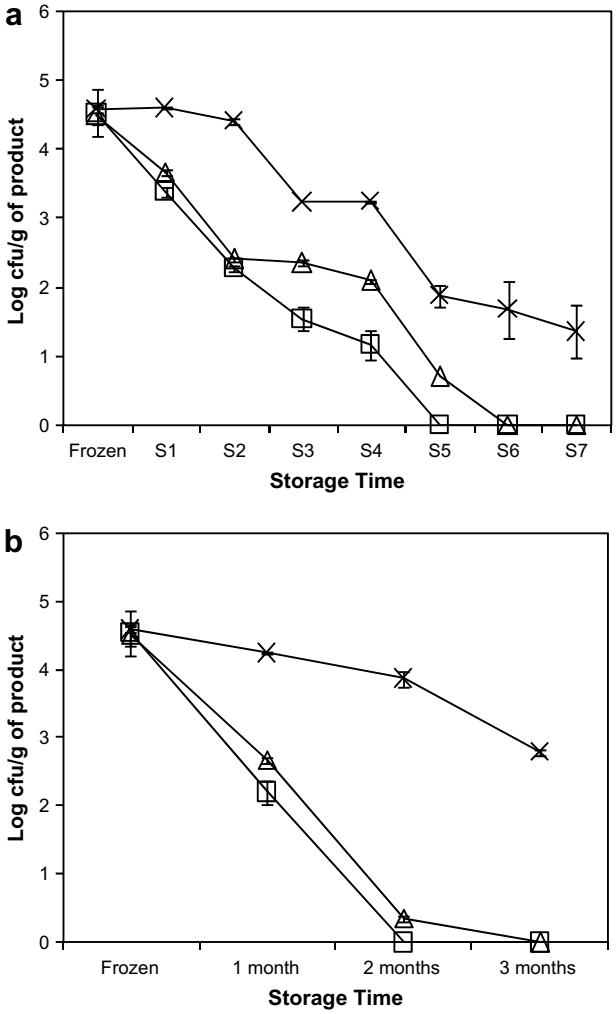

Fig. 2 – *Enterococcus faecalis* ATCC 29212 and *Pseudomonas aeruginosa* ATCC 27853 behaviour in artificially contaminated watercress: a) submitted to temperature abuses, according to pre-established plan (Fig. 1). b) During 3 months storage at −21 °C. The number of viable Enterococci (△), Pseudomonas (□) and TVC (×) were determined. Bars represent mean ± standard deviation.

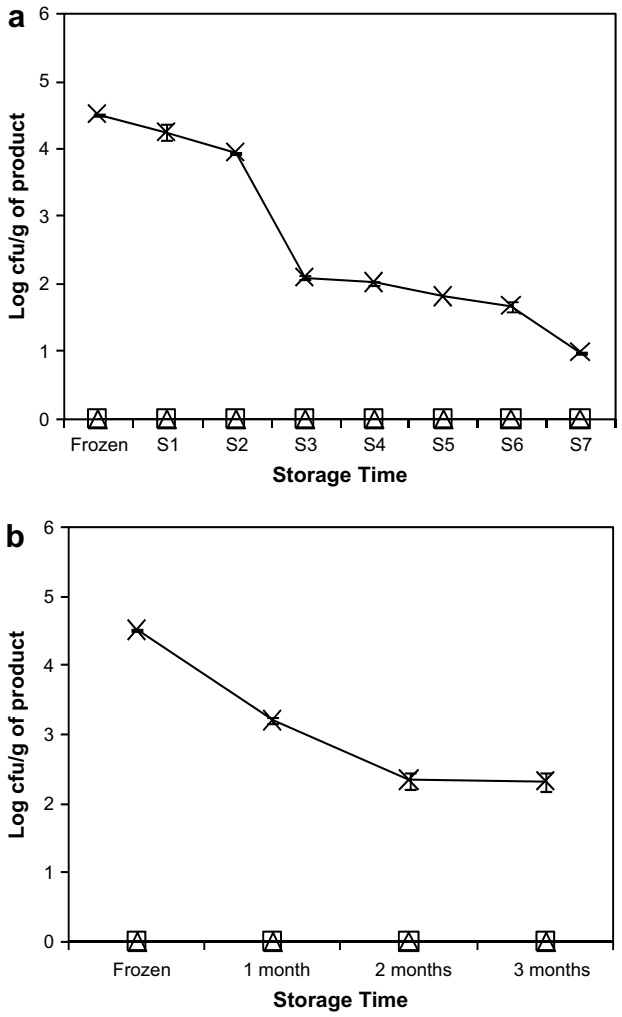

Fig. 3 – Microflora behaviour in non-contaminated watercress: a) submitted to temperature abuses, according to pre-established plan (Fig. 1). b) During 3 months storage at −21 °C. The number of viable Enterococci (△), Pseudomonas (□) and TVC (×) were determined. Bars represent mean ± standard deviation.

Along frozen storage at −21 °C, a TVC reduction of about 2 log cfu per gram of product was observed. Once again, the absence of *E. faecalis* and *P. aeruginosa* was confirmed along storage (Fig. 3b).

There were no significant differences ($P > 0.05$) in the reduction of TVC of non-contaminated and artificially contaminated frozen watercress, when submitted to temperature abuses (Figs. 2a and 3a). For both samples the reduction was about 3 log cfu per gram of product. Similar conclusion was obtained under constant storage temperature conditions (Figs. 2b and 3b).

Discussion

The results suggest that unlike *E. faecalis*, *P. aeruginosa* is a natural occurring microorganism in the studied watercress. Correa et al. (1991) reported the presence of *P. aeruginosa* in fresh watercress and concluded that it can represent a source of endemic infection for hospitalized patients. In a study with minimally processed watercress, Martins et al. (2004) also reported the presence of *Pseudomonas* spp. in watercress. However, the applied blanching/freezing processes killed or injured this pathogenic bacterium.

The net result of freezing and frozen storage is cellular damage, which accumulates over time. At some point the damage becomes irreparable and the cell will succumb when thawed. The process of partial thawing that occurs due to temperature fluctuations may exert more damage, since ice crystals grow (El-Kest and Marth, 1992). This occurs because thawing generally takes longer than freezing, and food remains longer in the subfreezing temperature zone, which favours recrystallization when small ice crystals melt and refreeze on larger ice crystals (Potter, 1986). At molecular level, freezing damage may be due to either dehydration or to the high intracellular solute and ionic concentrations, which result from the partial freezing of the cell water (Rudolph et al., 1986), effects similar to those which occur during osmotic dehydration. However, it has been estimated that at least 5% of cell water is unfreezable (Van Laere, 1989). Nevertheless, the natural occurring microorganisms seemed to be quite resistant to freeze–thaw cycles.

In a previous study (Cruz et al., 2003), watercress colour and vitamin C were also investigated. The results showed that despite the presence of sinusoidal fluctuations in these quality parameters due to the temperature abuses, their amplitude (positive or negative difference around the values right after freezing storage started) had no significant trend to be considered as a sign of impaired quality. Thus, it is possible to assume that frozen watercress quality preservation (colour and vitamin C) and safety can be achieved, regarding these temperature abuses.

The lack of studies, particularly on the impact of freezing storage fluctuations in the studied microorganisms, enhances the relevance of this work outcome, mainly when the occurrence of pathogens in frozen foods is a subject of concern to the food industry, consumers and the responsible health authorities.

Conclusions

At the end of the study it was concluded that the imposed freeze–thaw cycles led to the death or injury of the microorganisms. These findings allow understanding the effect of the freezing storage conditions on the behaviour of two concerning pathogens in frozen watercress.

This work proves that the use of the freezing process together with these freezing storage fluctuations in watercress, act as good barriers to food-borne pathogens and are of extreme relevance in public health since they will help improving the safety and cold chain settings of the frozen watercress. In conclusion, the watercress frozen slabs stability represents a valuable storage advantage for distributors and retailers, and also a convenient and healthy option to the final consumer.

Acknowledgements

The authors acknowledge support from Fundação para a Ciência e a Tecnologia (FCT), from Ministério da Ciência e do Ensino Superior, through project POCTI/43624/BIO/2000 and the Vitacress Company who kindly provided the fresh watercress.

The author Rui M. S. Cruz gratefully acknowledges his Ph.D. grant SFRH/BD/9172/2002 to Fundação para a Ciência e a Tecnologia (FCT) from Ministério da Ciência e do Ensino Superior.

REFERENCES

Archer, D., 2004. Freezing: an underutilized food safety technology? Int. J. Food Microbiol. 90, 127–138.

Arthey, D., 1995. Freezing of vegetables and fruits. In: Mallet, C.P. (Ed.), Frozen Food Technology. Blackie Academic and Professional, London, pp. 237–269.

Correa, C.M.C., Tibana, A., Gontijo Filho, P.P., 1991. Vegetables as a source of infection with *Pseudomonas aeruginosa* in a University and Oncology Hospital of Rio de Janeiro. J. Hosp. Infect. 18 (4), 301–306.

Cruz, R.M.S., Oliveira, S.R., Figueira, A.C., Silva, C.L.M., Vieira, M.C., 2003. Effect of temperature abuses on the quality of frozen watercress (*Nasturtium officinale*) during the cold chain. In: Proc. IFT Annual Meeting, Chicago, p. 242.

Cruz, R.M.S., Vieira, M.C., Silva, C.L.M., 2006. Effect of heat and thermosonication treatments on peroxidase inactivation kinetics in watercress (*Nasturtium officinale*). J. Food Eng. 72 (1), 8–15.

El-Kest, S.E., Marth, E.H., 1992. Freezing of *Listeria monocytogenes* and other microorganism: a review. J. Food Prot. 55, 639–648.

Geiges, O., 1996. Microbial processes in frozen food. Adv. Space. Res. 18 (12), 109–118.

Gram, L., Ravn, L., Rasch, M., Bruhn, J.B., Christensen, A.B., Givskov, M., 2002. Food spoilage-interactions between food spoilage bacteria. Int. J. Food Microbiol. 78, 79–97.

Liao, C.-H., 1989. Analyses of pectate lyases produced by soft rot bacteria associated with spoiling vegetables. Appl. Environ. Microbiol. 55, 1677–1683.

Liao, C.-H., Sullivan, J., Grady, J., Wong, L.-J.C., 1997. Biochemical characterization of pectate lyases produced by fluorescent pseudomonads associated with spoilage of fresh fruits and vegetables. J. Appl. Microbiol. 83, 10–16.

Lund, B.M., 1992. Ecosystems in vegetable foods. J. Appl. Bacteriol. Symp. Suppl. 73, 115S–126S.

Lund, B.M., 2000. Freezing. In: Lund, B.M., Baird Parker, T.C., Gould, G.W. (Eds.), The Microbiological Safety and Quality of Food. Aspen Publishers, Gaithersburg, MD, pp. 122–145.

Martins, C.G., Behrens, J.H., Destro, M.T., Franco, B.D.G.M., Vizeu, D.M., Hutzler, B., Landgraf, M., 2004. Gamma radiation in the reduction of *Salmonella* spp. inoculated on minimally processed watercress (*Nasturtium officinalis*). Radiat. Phys. Chem. 71, 87–91.

Nguyen-The, C., Prunier, J.P., 1989. Involvement of pseudomonads in deterioration of ''ready-to-use salads''. Int. J. Food Sci. Technol. 24, 47–58.

NP-1409/1987. Frutos, produtos hortícolas e seus derivados. Contagem de microrganismos a 30 °C.

Potter, N.N., 1986. Cold preservation and processing. In: Potter, N.N. (Ed.), Food Science. AVI Publishing, Westport, CT, pp. 201–245.

Roberts, D., Hooper, W., Greenwood, M., 1995. Isolation and enrichment of microorganisms. In: Practical Food Microbiology. Public Health Laboratory Service Publishers, pp. 143–144, 149–150.

Rudolph, A.S., Crowe, J.H., Crowe, L.M., 1986. Effects of three stabilising agents – proline, betaine and trehalose – on membrane phospholipids. Arch. Biochem. Biophys. 245, 34–143.

Van Laere, A., 1989. Trehalose, reserve and/or stress metabolite. FEMS Microbiol. Rev. 63, 201–210.