EVALUATION OF SURVIVAL PATTERNS AND CELLULAR INJURY OF *PSEUDOMONAS AERUGINOSA* IN DIFFERENT BOTTLED WATERS STORED UNDER VARIOUS CONDITIONS

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ABSTRACT

Pseudomonas aeruginosa *cells were inoculated into different waters and sampled after different periods of starvation in order to evaluate the influences of storage under daylight or dark conditions, the presence or absence of the autochthonous flora, the chemical composition of the water and the storage temperature, on survival. Survival was investigated by plate counts on selective and nonselective agar media. Light, low temperature (4C) and presence of the autochthonous flora negatively influenced the survival of* P. aeruginosa *during starvation in water. Higher survival rates were observed in waters with high mineral content. During starvation, cells developed sensitivity to the selective medium demonstrating that research is needed in the development of new media, or improvement in the existing ones, for the enumeration of* P. aeruginosa *in water. Current selective media/methodologies for detecting* P. aeruginosa *in mineral waters may seriously underestimate the levels of or presence of this organism which might represent, in some cases, a hazard to the public health.*

INTRODUCTION

The microbial quality of natural mineral water is of great interest since many consumers use it as an alternative to municipal water and consider it to be better and safer (Warburton 2000b). Within the EU, the marketing of natural waters is governed by a Council Directive (Anon. 1980). According to this Directive, treatment of mineral water destined for bottling is limited and, in particular, any treatment likely to change the viable colony count of the natural mineral water is not allowed.

Various studies have investigated the survival of pathogens when deliberately inoculated into bottled waters (Karapinar and Gönül 1991; Moreira *et al.* 1994; Kersters *et al.* 1996; Tamagnini and González 1997; Warburton *et al.* 1998; Kerr *et al.* 1999; Legnani *et al.* 1999; Ramalho *et al.* 2001). Different patterns of survival may be due to the different techniques used between laboratories, different strains of the organisms tested and different chemical compositions of the waters. Pathogenic bacteria might be present in water in an injured state due to exposure to suboptimal temperature, salinity, toxic chemicals or by starvation (McKay 1992). Injury may result in an increased sensitivity to selective agents such as antibiotics and surface active agents (Mackey 2000), and cells may also develop a requirement for some specific compounds for repair and subsequent growth. The use of selective media for the detection or enumeration of pathogens under stress conditions such as those impaired by the aquatic environment may therefore lead to their underestimation which might represent, in some cases, a serious hazard to the public health.

Pseudomonas aeruginosa is an important opportunistic pathogen, particularly to immunocompromised individuals. This organism expresses virulence factors which are related to serious infections in humans, especially in immunocompromised individuals. These factors include exotoxins, a phagocytosis resistant slime layer, and various enzymes and hemolysins that degrade host tissues (Bodey *et al.* 1983; Vachée and Leclerc 1995).The organism is widespread in natural and industrial environments, is able to grow in water (Vachée and Leclerc 1995) but cannot be used as an indicator of fecal contamination since it is not invariably present in feces and sewage. Examination of mains (piped) drinking water for *P. aeruginosa*, therefore, is not recommended as a routine procedure. However, it is of value in assessing the quality of bottled and mineral waters as an indicator of hygiene in the bottling plant. According to the European Directive for mineral waters (Anon. 1980), a natural mineral water shall be free of *P. aeruginosa* in any 250 mL sample.

Given the possibility of *P. aeruginosa* being a contaminant of bottled waters, batches or brands of bottled water in which these organisms have been detected have been withdrawn from retail outlets in Europe (Manaia *et al.* 1990). The long-term survival potential of *P. aeruginosa* in bottled waters and knowledge of its behavior under starvation conditions, may lead to new measures for its elimination. Several authors have studied the behavior of *P. aeruginosa* in bottled waters (Gonzalez *et al.* 1987; Tamagnini and González 1997; Legnani *et al.* 1999) but none, to our knowledge, has considered in an integrated study the influence of the various storage conditions and the chemical composition of the water. The purpose of the present study was to determine the behavior of *P. aeruginosa*, as an indicator of hygiene in bottling plants and a model pathogenic organism, during starvation in bottled waters of different compositions and stored under different conditions. The influences of storage under daylight or dark conditions, the storage temperature, the presence or absence of the autochthonous flora and the chemical composition of the water, on the survival of *P. aeruginosa* during starvation, were investigated. The ability of the organism to grow on selective media after starvation in bottled waters was also studied.

MATERIALS AND METHODS

Water Samples

Three mineral waters were tested. Waters A and B were sent from two bottling plants both in Central Portugal. Water C was obtained from the market. The chemical compositions of the water samples are presented in Table 1.

Culture Preparation

P. aeruginosa (NE 50) originally isolated from water and supplied by the culture collection of Escola Superior de Biotecnologia was used. Bacteria were grown in Brain Heart Infusion (BHI) broth (LAB M, Amersham, Bury, England) at 42C for 24 h. A stationary phase culture was harvested by centrifugation, and the cells washed three times by suspension and centrifugation with filtered (0.22 μm surfactant-free cellulose acetate syringe filters, Nalgene, New York) mineral water (respective to that to be inoculated). The cells were resuspended in filtered mineral water to the original culture volume.

Starvation

One mL of this suspension was then added to 0.33 l PET bottles (supplied by the bottling plant immediately after blowing at high temperature from a blank) previously rinsed and filled with one of the following water samples:

Water A; Filtered Water A (0.22 μm surfactant-free cellulose acetate syringe filters, Nalgene); Pasteurized Water A (30 min at 60C); Filtered Water B, or Filtered Water C.

TABLE 1.
CHEMICAL COMPOSITION (mg/L) OF THE MINERAL WATERS AS INDICATED BY THE SUPPLIERS

	Water A	Water B	Water C
	pH 5.7	pH 6.6	pH 7.2
SiO_2	13.3	16.0	
Cl^-	8.9	16	87
HCO_3^-	9.0	89	377
SO_4^{2-}	1.4		17.7
NO_3^-	1.7	0.5	
Na^+	6.3	0.5	39
Mg^{2+}	1.6	9.0	30.7
Ca^{2+}	0.7	12	103
Total mineralization	43.9	163	508

After being well shaken, the bottles were aseptically sampled immediately after the addition of the suspension and at regular intervals during storage in a water bath at 20C in daylight (ambient laboratory light, not direct sunlight) conditions. Water A was also stored in dark conditions (wrapped in aluminum foil) in water baths at 4 and 20C.

Uninoculated bottles of Water A were used to follow the numbers of the authochthonous flora during storage.

Viability Determinations

Serial decimal dilutions of samples were made in Ringer's solution and viable counts were determined by the spread plate technique on duplicate plates of Pseudomonas Agar Base (Pa; LAB M) with C.N. (cetrimide, nalidixic acid) selective supplement (LAB M). Viable counts in Water A were additionally determined by the spread plate technique on plates of BHI agar (LAB M). The plates were incubated at 42C for 24 h in order to prevent the growth of the autochthonous flora on BHI agar.

The autochthonous flora of the uninoculated Water A was enumerated by preparing decimal dilutions in Ringer's solution. Dilutions were spread plated on R2A Agar (Difco, Surrey, England), and incubated at 22C for 72h.

Results are representative of at least two independent experiments, which showed no more than 10-fold variability between equivalent time points.

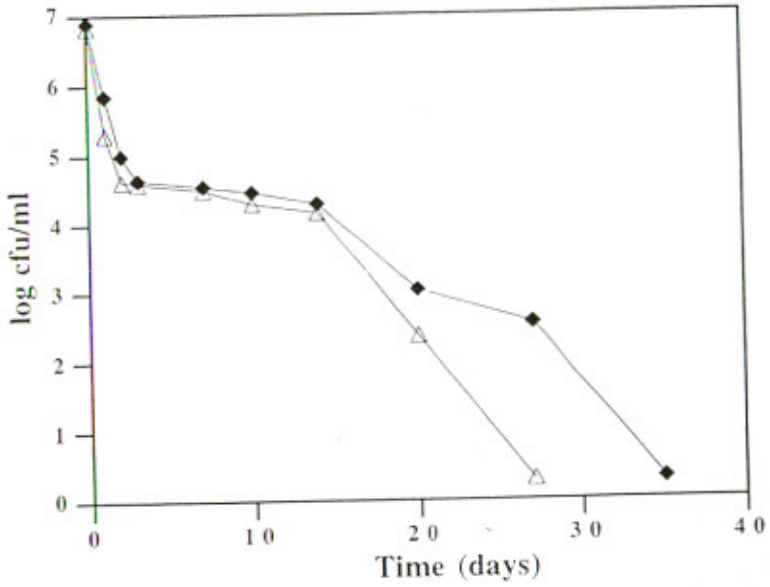

FIG. 1. SURVIVAL OF *PSEUDOMONAS AERUGINOSA* IN WATER A, STORED AT 20C IN DAYLIGHT CONDITIONS, WHEN ENUMERATION WAS PERFORMED ON NONSELECTIVE (♦) AND SELECTIVE (Δ) MEDIA

RESULTS

The population of *P. aeruginosa* decreased rapidly during the first 3 days of starvation in the dark in Water A and then the viable counts remained constant during the next 14 days. After this period, *P. aeruginosa* was rapidly inactivated

resulting in very low viable counts after 27 days or 35 days of storage when enumeration was performed on Pa or BHI, respectively (Fig. 1). Initial counts, before starvation, and during the first 14 days, were the same in selective and non-selective media. Thereafter, starved cells became progressively more sensitive to the selective agents in Pa (Fig. 1).

The storage of inoculated Water A in daylight conditions resulted in a greater loss of viability than during storage under dark conditions (Fig. 2).

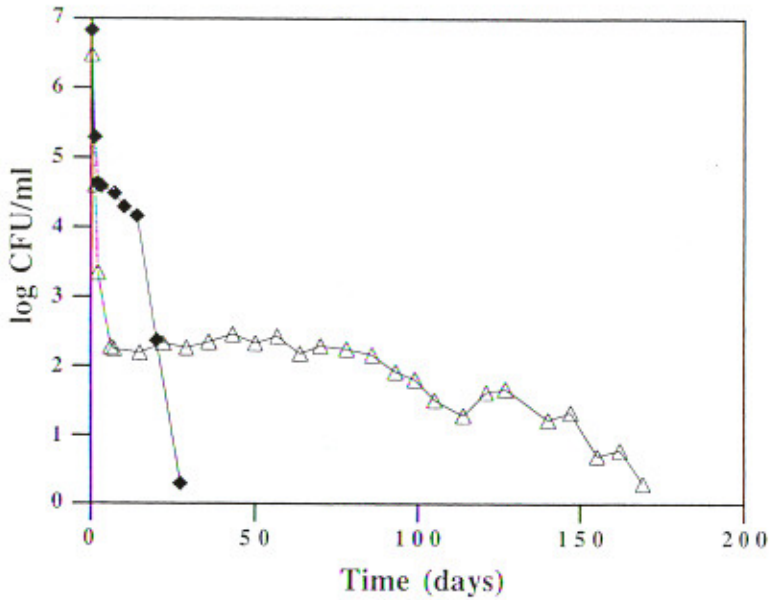

FIG. 2. SURVIVAL OF *PSEUDOMONAS AERUGINOSA* IN WATER A STORED AT 20C IN DAYLIGHT (♦) AND DARK CONDITIONS (Δ) WHEN ENUMERATION WAS PERFORMED ON SELECTIVE MEDIA

Higher survival rates were observed in filtered Waters B and C than in filtered Water A (Fig. 3). In filtered Water A there was a sharp decrease during the first 2 days of starvation. In filtered Water B, the organism increased by ca. 0.6 log CFU/mL during the first 15 days of starvation, then there was a sharp decrease between day 15 and day 70. In filtered Water C, the numbers of *P. aeruginosa* detected on the selective agar, after a small decrease, remained constant until day 65. Following this period, numbers decreased sharply until day 99. In these three waters following the initial sharp decrease, successive periods of growth and death were observed (Fig. 3).

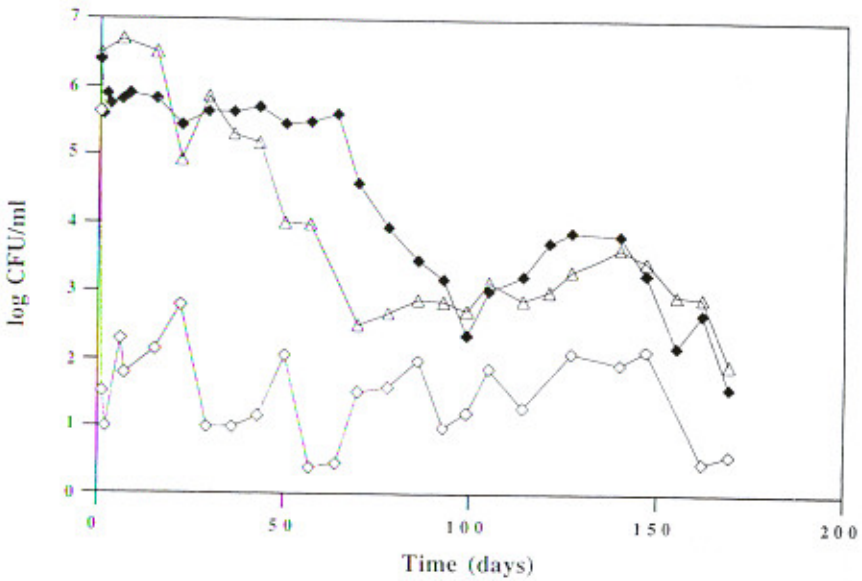

FIG. 3. SURVIVAL OF *PSEUDOMONAS AERUGINOSA* IN FILTERED WATER A (◊), FILTERED WATER B (Δ), AND FILTERED WATER C (♦) DURING STORAGE AT 20C IN DAYLIGHT CONDITIONS WHEN ENUMERATION WAS PERFORMED ON SELECTIVE MEDIA

P. aeruginosa survived for longer periods in filtered and pasteurized than in nontreated Water A (Fig. 4). Initially, the number of autochthonous bacteria in uninoculated Water A was approximately 1000 CFU/mL increasing to 10^5 CFU/mL after seven days of storage. During the rest of the storage period, the number of bacteria remained more or less constant (Fig. 4). In the absence of the autochthonous flora *P. aeruginosa* increased in numbers considerably (log 1 to log 5 CFU/mL) after an initial sharp decrease (log 6 to log 1 CFU/mL). This growth was much greater in pasteurized than in filtered water. Following this period of growth, another sharp decrease was observed in pasteurized water (log 5 to log 0.5 CFU/mL) and a smaller decrease in filtered water (log 2.7 to log 1 CFU/mL), and then successive periods of growth and death were observed in both types of treated Water A (Fig. 4).

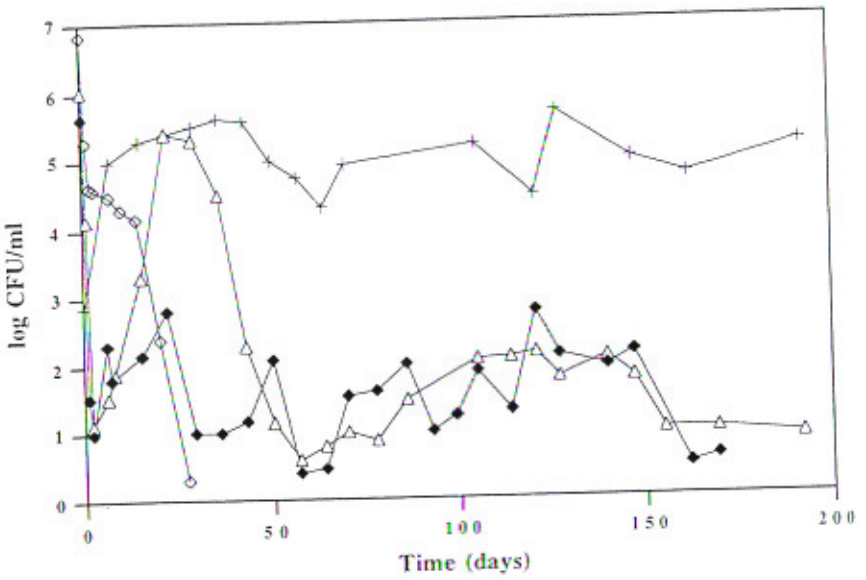

FIG. 4. SURVIVAL OF *PSEUDOMONAS AERUGINOSA* IN WATER A (◊), PASTEURIZED WATER A (Δ) AND FILTERED WATER A (♦) STORED AT 20C IN DAYLIGHT CONDITIONS WHEN ENUMERATION WAS PERFORMED ON SELECTIVE MEDIA

Viability of the autochthonous bacteria in water A (+) during storage at 20C in daylight conditions.

The effect of temperature on the survival of *P. aeruginosa* during starvation in Water A is illustrated in Fig. 5. Cells survived for a longer period in water stored at 20C than at 4C.

DISCUSSION

In order to assess to what extent the presence of *P. aeruginosa* may pose a health risk, it is necessary to determine the survival of this organism in different waters and under different conditions of storage.

As viability on nonselective and selective media before starvation showed no significant differences from one another, the differences observed after 14 days of starvation using the same growth media can be safely attributed to sublethal

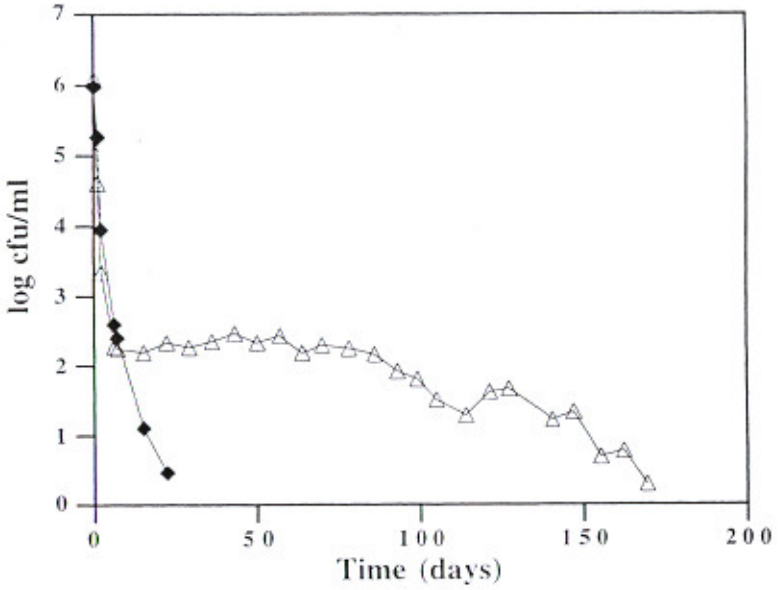

FIG. 5. SURVIVAL OF *PSEUDOMONAS AERUGINOSA* IN WATER A STORED AT 4C (♦) AND 20C (Δ) IN DARK CONDITIONS WHEN ENUMERATION WAS PERFORMED SELECTIVE MEDIA

lesions in a large proportion of the population induced by stress factors in water. The addition of C.N. supplement (200 mg/L cetrimide, 15 mg/L nalidixic acid) to Pa makes the medium selective for *P. aeruginosa* (Goto and Enomoto 1970). Previous studies demonstrated that during starvation *P. aeruginosa* becomes sensitive to cetrimide but not to nalidixic acid (Ramalho *et al.* unpublished observations). Cetrimide, a quaternary ammonium compound, apparently acts by disrupting the bacterial cell membrane with a resulting increase in permeability. Gram-negative bacteria are generally less susceptible to biocides than Gram-positive species, mainly because the outer membrane acts as a protective barrier. Cetrimide is generally not active against *P. aeruginosa*. Results obtained, however, showed that after starvation cells become sensitive to this compound probably as a result of outer membrane damage. The presence of injured bacteria in water has already been reported (Bissonette *et al.* 1975; McFeters *et al.* 1986) and the cell envelope was considered a major site of injury in water-injured coliforms (Zaske *et al.* 1980). This work demonstrates the need for research in the development of new media, or improvement in the existing ones, for the detection and enumeration

of damaged cells of *P. aeruginosa* in water. Recommendations for the use of resuscitation broths in screening bottled water for the presence of indicator and pathogenic bacteria have already been presented by Warburton (2000a). Use of the Pa medium, or other selective media containing cetrimide, could give rise to a false impression of the safety of a sample of bottled water.

An initial sharp decrease in the number of *P. aeruginosa* survivors in water had already been recorded by other workers (Moreira *et al.* 1994) and of other allochthonous organisms (Kersters *et al.* 1996). This has been attributed to a requirement for a period of physiological adaptation to stress conditions (Matin *et al.* 1989; Roszak and Colwell 1987) after which the mortality rate decreases. Watson *et al.* (1998) observed that during starvation in water, about 99% of *Staphylococcus aureus* cells lost viability within the first two days and the viability of the culture then remained relatively constant for a period of months. This was attributed to cryptic growth, where most of the cells die, providing nutrients for the maintenance of the remaining surviving cells (Mossel 1976; Schmidt-Lorenz 1976). Different observations, however, were made by Vachée and Leclerc (1995), when the number of *P. aeruginosa* remained stable for 9 months in a mineral water, whereas in bottled spring water Tamagnini and González (1997) and Legnani *et al.* (1999), reported that *P. aeruginosa* increased in numbers. In the study by Tamagnini and González (1997) the samples were stored in the dark, whereas a destructive effect of light on the viability of *P. aeruginosa* during starvation in water was recorded in the current study and had already been reported for other organisms in aquatic systems (Reed 1997). This destructive effect of light was attributed to the production of reactive forms of oxygen, mainly hydrogen peroxide and hydroxyl radicals (Arana *et al.* 1992; Gourmelon *et al.* 1994). An alternative explanation for the different results, since the initial sharp decrease in the survivors was observed even when storage was in dark conditions, was that differences in the chemical composition or in the organic carbon content of the waters may be influential. According to Warburton and Austin (2000), generally, waters having a high content of minerals support bacterial growth for long periods of time, as opposed to distilled water. The role of calcium and magnesium in the stabilization of various cellular structures is very well known. When *P. aeruginosa* was inoculated into waters with a high mineral content (Waters B and C) the initial sharp decrease in culturability was not observed. In their study, Tamagnini and González (1997) used a highly mineralized water, whereas Legnani *et al.* (1999) found no differences in the survival of *P. aeruginosa* in high or low mineralized water. Vachée and Leclerc (1995) did not describe the composition of the water used in their experiments. However, Legnani *et al.* (1999) heated their water samples at 75C for 15 min before inoculating with *P. aeruginosa*. As demonstrated in this study, *P. aeruginosa* survived for a longer period in the absence than in the presence of the autochthonous flora. In the absence of the autochthonous flora, *P. aeruginosa* was even able to grow. More growth was observed in pasteurized water than in filtered water probably because components of cells killed by the heat treatment were used as substrates for growth by *P. aeruginosa* in this water. Only in the case of filtered water did *P. aeruginosa* cells that died provide nutrients for growth of surviving cells. Moreira *et al.* (1994) and Vachée and Leclerc (1995) also observed that the autochthonous flora had a negative effect on the survival of *P. aeruginosa* in water. Tamagnini and González (1997) observed that *P. aeruginosa* had a longer doubling time in water in the presence than in the absence of the autochthonous bacteria. The antagonistic effect of the autochthonous flora towards allochthonous microorganisms has previously been reported (Karapinar and Gönül 1991). The mechanism responsible for this antagonistic effect of the autochthonous flora of mineral water on *P. aeruginosa* and other microorganisms, remains unknown.

Greater survival of *P. aeruginosa* was observed in water stored at 20 than at 4C, common temperatures under which bottled waters are stored by consumers. Gonzales *et al.* (1987) showed that *P. aeruginosa* grew in water at 20, 30 and 37C, whereas at 6C it was rapidly inactivated. This was attributed to the higher growth rate of the autochthonous flora at low temperatures than at room temperature. In this study it was not possible to count the autochthonous flora in inoculated waters since *P. aeruginosa* can grow in the same media and incubation conditions. It seems unlikely that lower survival at 4 than at 20C was related with greater growth of the autochthonous flora at the lower temperature. Schmidt-Lorenz *et al.* (1990) showed that 25 isolates from mineral water had an optimum temperature for growth in diluted broth of 20 to 32C and other workers have demonstrated that during storage at 4C the autochthonous flora grew in bottled water at a lower rate than at 20C although they attained the same final number (Moreau and Thomassey, unpublished observations). Another possibility might be a lower enumeration efficiency of the *P. aeruginosa* cells stored at 4C due to the temperature shock existing between storage (4C) and incubation temperatures (42C) of plates.

Bottled water sold worldwide has generally been found to be of good microbiological quality (Warburton 2000b). However, given the possibility of growth of *P. aeruginosa* in bottled water, as demonstrated in this study, consumers should be advised, as a minimum safety precaution, to refrigerate it upon opening.

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