Thermal inactivation of *Listeria monocytogenes* from *alheiras*, traditional Portuguese sausage during cooking

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A B S T R A C T

D values were calculated at 55, 60 and 65 °C for five *Listeria monocytogenes* isolates using *alheira* (traditional Portuguese sausage) food matrix as heating menstrum. *z* values were also calculated and combined with internal *alheiras* temperature profiles during cooking in multiple ways, allowed estimations of percentages of *L. monocytogenes* survival.

Survival percentages estimations showed that except for roasting, the remaining evaluated cooking methods might not be sufficient to inactivate this foodborne pathogen in *alheiras* at the minimum temperature profiles. However, it is important to note that all evaluated cooking methods were able to inactivate *L. monocytogenes* in *alheiras* at their maximum internal temperature profiles.

Keywords:
Listeria monocytogenes
Thermal inactivation
Cooking
Alheira

1. Introduction

The origin of *alheiras* goes back to the fifteenth century during the Inquisition period when Jews devised a type of sausage that would give the appearance of being made with pork, but really contained a variety of meats, including heavily spiced game (such as partridge) and poultry. Nowadays, however, pork as well as other types of meats (calf and beef), are added to these traditional smoked fermented sausages. *Alheiras* are produced using traditional methods of boiling small chunks of these meats in water with salt and spices. Thinly sliced bread is then soaked in an aliquot of the broth from the boiled meat. Next, the boiled meats, spices, olive oil and/or fat drippings are added to the bread/broth mixture. After thorough mixing, a paste is formed and stuffed in either pork intestines or cellulose casings for the production of sausages. The sausages are subsequently smoked, typically between 2 and 8 days.

Alheira sausages represent an important part of the daily diet in rural parts of Portugal but are also becoming increasingly popular in urban areas. For example, annual production of *alheiras* in the Mirandela region ("*alheiras de Mirandela*") has been increasing since 1998 reaching an annual production of 136 tons in 2003. *Alheiras* account for approximately 60% of the total tons of meat products produced in Portugal (Oliveira, 2004, 2005). Costing only about 4.5 euros/kg, *alheiras* are the cheapest meat product available; whereas expensive meat products, such as *Salpicão de Vinhais*, can reach prices of 37.5 euros/kg (Oliveira, 2005).

A preliminary microbiological characterization of *alheiras* by Ferreira et al. (2007) demonstrated that more than 60% of the analyzed lots that were classified as potentially hazardous were contaminated with *Listeria monocytogenes* at levels higher than 100 cfu/g pH and a_w (ranging from 4.1 to 5.3 and 0.96 to 0.98 respectively) *per se* do not assure the microbiological safety of this product. The meats in this product are boiled sufficiently to inactivate the vegetative pathogens, but post-process contamination can occur via the addition of the bread and spices or by subsequent handling during filling and subsequent manipulations.

Consumers usually grill, roast, fry, boil or even microwave *alheiras* before consumption. Hence *alheiras* are generally not considered RTE foods. Storage of *alheiras* at refrigeration temperatures allows *L. monocytogenes* to grow and insufficient cooking of these products might represent an important food safety issue. Furthermore, locally produced and artisanal foods may represent potentially important sources of human listeriosis cases, as supported by a large listeriosis outbreak in France (279 cases) linked to consumption of pork tongue in jelly (Jacquet et al., 1995).

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The objectives of this work were to evaluate the thermal inactivation behavior of *L. monocytogenes* from *alheiras* and infer lethality based on the internal measured temperature profiles of *alheiras* during cooking using different methods, such as grilling, frying and roasting.

2. Materials and methods

2.1. Internal alheiras temperature measurements

Monitoring internal temperature profiles of *alheiras* during cooking in multiple ways, such as: frying, electric grilling and roasting in a gas oven as well as a wood-fired oven, was accomplished using an eight channel thermocouple datalogger 100 series squirrel (Grant Instruments, Cambridgeshire, U.K.). Eight welded tip thermocouples of type K (Chromel/Alumel) were calibrated by introduction into boiling water (100 °C) and ice (0 °C). Subsequently, the room temperature in the kitchen environment was measured and registered. Thermocouples were placed along the coldest spot of the *alheiras* i.e. the slowest heating point during a thermal process. The coldest spot is usually at the geometric center of the food being processed. Therefore thermocouples were placed along the central line of the *alheiras* at 8 different locations (Fig. 1). Measurements were registered every 10 s and each recorded value corresponded to the average temperature value between two readings taken at 5 s time intervals.

2.2. Thermal inactivation experiments

2.2.1. Selection and preparation of inocula

Five *L. monocytogenes* isolates from alheiras were selected from the culture collection of Escola Superior de Biotecnologia (Porto, Portugal) for the thermal inactivation experiments. One isolate was selected per identified serogroup, i.e. FSL-F7-020 for serotypes 1/2a and 3a, FSL-F7-088 for serotypes 1/2c and 3c, FSL-F7-099 for serotypes 4b, 4d, and 4e and FSL-F7-128 for serotypes 1/2b, 3b, and 7. Isolates were chosen to represent the most prevalent combined PFGE pattern, which was found among the highest number of producers. An additional virulence attenuated isolate was chosen, namely FSL-F7-001. All selected isolates were streaked on TSAYE. To prepare each pre-inoculum, a colony of every isolate was transferred to TSBYE in a test tube that was incubated overnight at 37 °C. This cell suspension was subsequently diluted at 1:100 in TSBYE and incubated at 37 °C for 18 h to yield stationary phase cultures. The obtained inoculum subcultures were centrifuged at 5000 rpm for 10 min at 4 °C and the pellet was resuspended in half of the initial volume with sterile Ringer's solution.

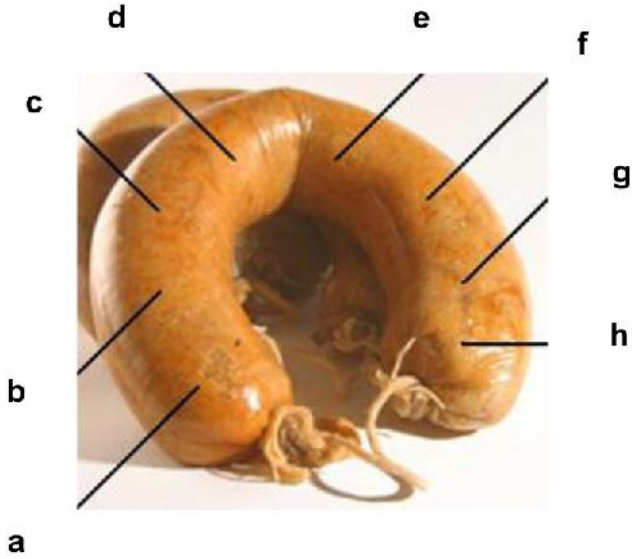

Fig. 1. Schematic representation of the 8 locations per *alheira* used for internal temperature measurement. Letters a to h represent the places along the central line of the *alheiras* i.e. the slowest heating point, where temperature was measured every 10 s throughout cooking.

2.2.2. Thermal inactivation experiments in alheira food matrix

The study of the inactivation kinetics of the *L. monocytogenes* isolates was performed at three different temperatures, namely 55, 60 and 65 °C. *Alheira* food matrix was used as heating menstrum. Samples of one g of *alheira* were distributed into multiple test tubes and sterilized by autoclaving at 121 °C for 15 min. Subsequently, these test tubes were immersed in the water bath and equilibrated to the selected temperature, which was maintained throughout the experiments. *Alheira* samples were inoculated into the center with 100 μl of the cellular suspensions of tested inocula. At defined timepoints, test tubes with inoculated *alheira* samples were taken out of the water bath and immediately diluted in Ringer's solution (9.0 ml) at room temperature.

Heat treatments were carried out in a water bath model Precisterm (Selecta, Barcelona, Spain). Each experiment was repeated three times at each temperature. Viability of all tested isolates was verified at the beginning of each experiment by enumerating one sample of inoculated heating menstrum maintained at room temperature. Temperature monitoring was conducted throughout experiments using a datalogger 100 series squirrel (Grant Instruments, Cambridgeshire, U.K.) A welded tip thermocouple of type K (Chromel/Alumel) was placed into the tested non-inoculated heating menstrum inside a control glass flask or test tube. As soon as the required temperature was reached, inoculation of *alheira* samples inside test tubes was performed.

2.2.3. L. monocytogenes enumeration

Viable *L. monocytogenes* numbers in samples withdrawn at defined timepoints throughout the experiments were determined as follows. Samples were serially diluted and plated using the drop count technique (Miles & Misra, 1938) onto the non-selective culture medium TSAYE, which was used to enumerate both injured and non-injured *L. monocytogenes* cells. Briefly, two drops (20 μl each) of suitable dilutions were spot-inoculated on marked places on each agar plate without spreading. Plates were incubated at 37 °C for 48 h. Mean values of bacterial counts, from triplicate plate samples, were converted to log numbers for each heating time and tested temperature.

2.2.4. Calculation of D, z values and lethality of thermal processing

D values (decimal reduction time, or time required to inactivate 90% of the population) were calculated as the absolute value of the inverse slope of the least square regression line fitted to $\log_{10}$ reduction in viable cell numbers versus heating time (Rowan & Anderson, 1998). Linear regression lines were fitted to the linear portion(s) of the survival curve. Each survival curve was plotted considering mean bacterial counts from three replicates using TSAYE as recovery culture medium.

The *z* value is the temperature difference needed to reduce (or increase) the *D* value by a factor of 10. A linear regression was computed from $\log_{10} D$ value versus heating temperature, and the *z* value was calculated as the absolute value of the inverse of the slope (Casadei, Esteves De Matos, Harrison, & Gaze, 1998). Temperatures used to determine *z* values were 55, 60, and 65 °C.

The time and temperature dependence of thermal pathogen destruction can be described by using the well known concept of

the thermal reduction time (D) and the temperature difference required for the thermal inactivation curve to decrease by one logarithmic cycle, (z). Therefore, the *L. monocytogenes* thermal process lethality, (F), was calculated by the following equation (Murphy, Beard, Martin, Keener, & Osaili, 2004; Murphy, Duncan, Driscoll, & Marcy, 2003):

$$F = \int_0^t 10^{[T(t)-T(\mathrm{ref})]/z} \mathrm{d}\, t$$

where $T(t)$ was the temperature during a process at the *alheira*'s center at time t, T(ref) was the reference temperature (assumed to be the average temperature of the range studied, i.e. 60 °C) and z was the highest z value that was obtained among tested isolates representing the worst-case scenario. The temperature profiles ($T(t)$) selected for each cooking method were those where the minimum and maximum temperatures were attained, thus representing the worst- and best-case scenarios. *L. monocytogenes* survival ratios (N/N_0) were estimated from lethality calculations.

2.3. Statistical analysis

Data regarding D values and z values were analyzed for any significant differences between isolates by analysis of variance (ANOVA) and Duncan's multiple-range test at a 95% confidence level. Statistica 6.0 (StatSoft, Tulsa, OK, U.S.) or Microsoft® Excel 2000 (Microsoft Corporation, Redmond, WA, U.S.) were used for all calculations and statistical analysis procedures.

3. Results

3.1. Heat resistance of L. monocytogenes isolates from alheiras

D value has been traditionally treated as a measure of the organism's heat resistance in the particular medium in which the inactivation has been monitored (Peleg, 2006, p. 1). D values in *alheira* paste heated at 55, 60 and 65 °C were calculated for five *L. monocytogenes* isolates from *alheiras* (Table 1).

D values of *L. monocytogenes* isolates from *alheiras* ranged from 7.2 to 9.3, 1.3 to 2.1 and 0.6 to 1.8 min at 55, 60 and 65 °C respectively (Table 1). Confidence intervals at 95% were calculated for each D value and are shown in Fig. 2. R^2 for all determined D values ranged from 0.77 to 0.96 (Table 1). z values were also calculated for five *L. monocytogenes* isolates from *alheiras* and ranged from 8.9 to 15.8 °C with R^2 ranging from 0.81 to 0.95.

The heating profile of *alheiras* was monitored during real scenarios of cooking, namely: electric grilling at a private home, frying at a typical tavern as well as at the university's kitchen and roasting at a typical restaurant in a wood-fired oven and at the university's kitchen in a gas oven. An example of the minimum and maximum temperature profiles is represented for each cooking method in Figs. 3 and 4, respectively. In general, the internal temperature profiles varied to a great extent among thermocouples particularly when frying or grilling *alheiras*. Minimum internal temperature profiles of *alheiras* observed during frying or grilling suggest the persistence of cold spots throughout thermal processing. To be effective, a heat process needs to guarantee that the coldest point in the treated object has been exposed to a sufficiently high temperature for a sufficiently long time to cause the destruction of the targeted organism. This validates the ability of the process to guarantee the products' microbial safety in the worst-case scenario.

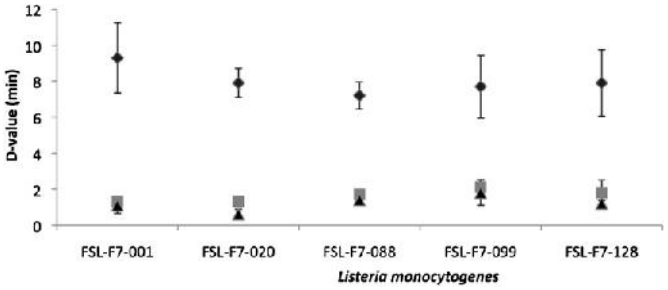

Fig. 2. D values at 55 (◆), 60 (■) and 65 (▲) °C and their confidence intervals at 95% (shown as y error bars) for five *Listeria monocytogenes* isolates from *alheiras*.

Therefore, in order to infer about the lethality of different methods of thermal processing of *alheiras* by consumers, minimum and maximum temperature profiles were used simulating worst- and best-case scenarios. Survival ratios (i.e. N/N_0) of *L. monocytogenes*, which were estimated, indicate that the attained minimum internal temperatures might not be sufficient to kill *L. monocytogenes* for some evaluated cooking methods (Table 2). Survival ratios of *L. monocytogenes* in *alheiras* that were fried or grilled ranged from 0.12 to 0.98 and 0.38 to 0.83 respectively (Table 2). Thus, except for roasting, the remaining evaluated cooking methods might not be sufficient to inactivate this foodborne pathogen in *alheiras* at the minimum temperatures attained. However, it is important to note that all evaluated cooking methods were able to inactivate *L. monocytogenes* in *alheiras* at their maximum internal temperatures attained.

4. Discussion

There have been several studies done to determine the D and z values of *L. monocytogenes* in various ground and whole-meat products. Thermal inactivation rates of *L. monocytogenes* at any given temperature varied considerably among studies and when the pathogen was heated in different media. $D_{60°C}$ values for most products are in the range of 1.8–8.3 min (Farber, Pagotto, & Scherf, 2007). Since no reports were found on D and z values of *L. monocytogenes* in *alheiras* or similar traditional meat products, it

Table 1
Heat resistance of *L. monocytogenes* in *alheira* paste during heating at 55 °C, 60 °C and 65 °C using as recovery culture medium.

Temperature	55 °C			60 °C			65 °C				
Isolate[a]	D value (min)	R^2	Confidence interval at 95°%	D value (min)	R^2	Confidence interval at 95°%	D value (min)	R^2	Confidence interval at 95°%	z value (°C)	R^2
FSL-F7-001	9.3	0.79	[7.3; 11.2]	1.3	0.92	[1.0; 1.5]	1.1	0.82	[0.6; 1.5]	10.8	0.81
FSL-F7-020	7.9	0.96	[7.1; 8.7]	1.3	0.95	[1.1; 1.5]	0.6	0.81	[0.4; 0.9]	8.9	0.95
FSL-F7-088	7.2	0.94	[6.5; 8.0]	1.7	0.96	[1.5; 1.8]	1.4	0.92	[1.1; 1.7]	12.2	0.91
FSL-F7-099	7.7	0.77	[5.9; 9.4]	2.1	0.89	[1.7; 2.5]	1.8	0.84	[1.1; 2.5]	15.8	0.83
FSL-F7-128	7.9	0.83	[6.0; 9.7]	1.8	0.85	[1.1; 2.5]	1.2	0.96	[1.1; 1.4]	12.2	0.90
Average ± s	8.0 ± 0.8	NA	NA	1.6 ± 0.3	NA	NA	1.2 ± 0.4	NA	NA	12.0 ± 2.5	NA

NA = not applicable.
[a] All strains were isolated from alheiras.

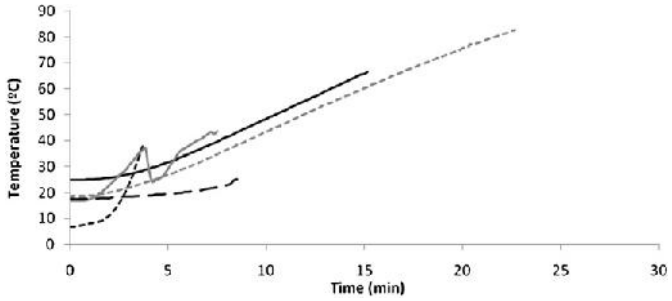

Fig. 3. Profiles of minimum internal temperatures of *alheiras* during cooking by the following methods: roasting in a typical restaurant with wood-fired oven (WFOmin, —), frying in a tavern (FTmin, —), electric grilling in a private home (GPHmin, —), roasting in the university's kitchen oven (RUmin, —), and frying in the university's kitchen (FUmin, — —).

is difficult to compare this study with previous publications. In general, the *D* and *z* values of *L. monocytogenes* in *alheiras* paste were of the same magnitude as those previously reviewed by Doyle, Mazotta, Wang, Wiseman, and Scott (2001) for ground meat and poultry and cured meats. It should be noted that some tailing of the survival curves was observed, particularly at higher temperatures, which may be due to a subpopulation of cells that are more heat resistant than the majority of the population (Miller, Brandão, Teixeira, & Silva, 2006).

Strain diversity in heat resistance was observed among *L. monocytogenes* isolates from *alheiras*. This is in accordance with literature data indicating that significant strain diversity in heat resistance can naturally exist among *L. monocytogenes* isolates with, for example, *D* values at 57 °C ranging from 6.5 to 36 min (Mackey, Pritchet, Norris, & Mead, 1990). Although the heat resistance of *L. monocytogenes* has been studied extensively, in most studies only one strain has been tested (Buncic, Avery, Rocourt, & Dimitrijevic, 2001). In the few studies where multiple isolates were investigated, *L. monocytogenes* showed strain diversity in heat resistance (De Jesus & Whiting, 2003; Lianou, Stopforth, Yoon, Wiedmann, & Sofos, 2006). However, particular heat resistance characteristics could not be linked to particular serotypes (Sörqvist, 1994). When only clinical isolates of *L. monocytogenes* were grouped together, the mean D_{60} value of serotype 4b was significantly higher than that of serotype 1/2a. In contrast, when only *L. monocytogenes* food isolates were grouped together, no significant difference in mean *D* values between the two serotypes was observed (Buncic et al., 2001).

Data from multiple studies were pooled and used to estimate a *z* value of 7.6 °C for *L. monocytogenes* (Lado & Yousef, 2007). A large number of published inactivation studies were reviewed and *D* and *z*

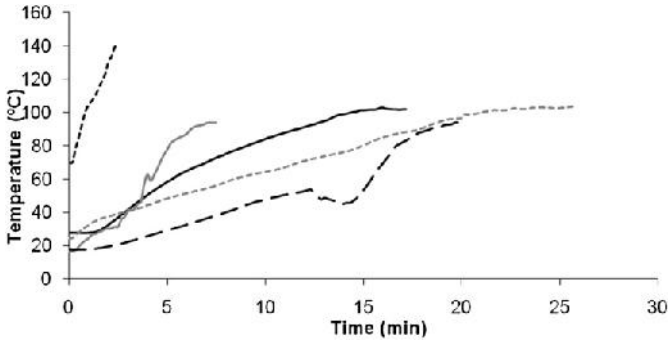

Fig. 4. Profiles of maximum internal temperatures of *alheiras* during cooking by the following methods: roasting in a typical restaurant with wood-fired oven (WFOmax, —), frying in a tavern (FTmax, —), electric grilling in a private home (GPHmax, —), roasting in the university's kitchen oven (RUmax, —), and frying in the university's kitchen (FUmax, — —).

Table 2
Survival rates of *L. monocytogenes* estimated based on process lethality at the highest and lowest internal temperature profiles of *alheiras* attained during various cooking methods.

Cooking method	Temperature profile *T(t)*	Survival rate [a] N/N_0
Roasting in wood-fired oven in typical restaurant (WFO)	maximum	0
		0
		0
	minimum	0
		0
		0
Roasting in university oven (RU)	maximum	0
		0
		0
	minimum	0
		0
		0
Grilling in private home (GPH)	maximum	0
		0
		0
	minimum	0
		0.38
		0.83
Frying in tavern (FT)	maximum	0
		0
		0
	minimum	0.98
		0.12
		0
Frying in university kitchen (FU)	maximum	0
		0
		0
	minimum	0.93
		0.42
		0.97

[a] Survival rates (N/N_0) were determined for three randomly chosen *alheiras* per cooking method.

values of *L. monocytogenes* were compared for various laboratory media and food products. The authors of this comparative review reported average *z* values of 7.1 °C (minimum: 4.3 °C; maximum: 29.3 °C) (Doyle et al., 2001). *z* values obtained for *L. monocytogenes* isolates from *alheiras* ranged from 8.9 to 15.8 °C. Differences in the extent of heat protection afforded by the environment in which heating takes place might explain variation among *L. monocytogenes* isolates. It should be noted that the *alheira* paste matrix is quite heterogenous and its composition and hence influence on heat resistance of *L. monocytogenes* are likely to vary widely between different parts of one product as well as between products from different processors.

The resistance of *L. monocytogenes* to mild thermal processes has frequently been observed. At temperatures < 60 °C, *Listeria* spp. had a substantially higher *D* value than *Salmonella* spp. in meat products, especially chicken (Murphy, Duncan, Johnson, Davis, & Smith, 2002). The thermal resistance of *L. monocytogenes* in meat is considerably higher than that of *Salmonella* which is often used to establish minimum thermal processing conditions. This higher thermal tolerance should be taken into consideration when developing processes designed to control pathogens in processed meats (Schoeni, Brunner, & Doyle, 1991).

Overall, a wide variability of the internal temperature profiles of *alheiras* was observed during cooking by different methods. In another study a wide variability was also observed in the internal temperature of beef hamburgers at the end of cooking (Passos & Kuaye, 2002). These variabilities can be due to heterogeneity of the food products, the complexity of the cooking process, as well as the difficulty of maintaining the thermocouple in the geometric center of the product during cooking. Some of the internal temperature profiles of *alheiras* observed during frying or grilling

were quite low and suggest the persistence of cold spots in these products throughout processing by the consumer. This might be due to insufficient thawing of frozen *alheiras*. Freezing of *alheiras* is a common practice at the tavern that allowed monitoring of internal temperatures of *alheiras* during frying. This might promote the development of cold spots throughout speedy frying. Roasting of *alheiras* (in a gas- or wood-fired oven) proved to be the only cooking method which guaranteed *L. monocytogenes* inactivation at the maximum as well as minimum temperature profiles. The conflicting results for heat resistance of *L. monocytogenes* in *alheiras* cooked in various ways raise questions about the validity of defining safe and universal cooking treatments.

When high levels of *L. monocytogenes* are present, this organism may grow during refrigerated storage (4 °C for at least 48 h) of ground beef and survive cooking (to an internal temperature up to 70 °C), and it can possibly reach high numbers during storage (Boyle, Sofos, & Schmidt, 1990). While *alheiras* are cooked before consumption either by frying, grilling roasting, boiling or microwaving according to regional traditions or consumer preferences, the temperatures achieved during cooking of *alheiras* by the consumer might also be insufficient to inactivate this foodborne pathogen. In this context *alheiras* could be included in the RTE foods category. In Europe, at present, microbiological criteria for *L. monocytogenes* are only defined for RTE foods. Therefore, the establishment of another food category could be included in the European microbiological criteria for foodstuffs, Commission Regulation No. 1441/2007. This category could be defined as foods that are able to support the growth of *L. monocytogenes* and undergo heat processing by the consumer that might not be effective to eliminate *L. monocytogenes*.

It is critical to advise consumers who fry or grill *alheiras*, to do so for a prolonged period in order to reduce the survival probability of *L. monocytogenes*, if present at all in this food product. Similarly, it would be reasonable to recommend labeling of *alheiras* with instructions on how to cook them (temperature and cooking time) and alerting consumers to the risks posed to those groups of the population such as pregnant women, infants, the elderly or immunocompromised individuals.

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