Majorensis 2011; **7**: 5-13 www.majorensis.es

Majorensis
ISSN 1697-5529

Behavior of *Escherichia coli* at low temperature in isothermal and non-isothermal conditions

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Summary
Behavior of *Escherichia coli* at low temperature in isothermal and non-isothermal conditions.
We analyzed the behavior of *Escherichia coli* at suboptimal growth temperature with strains isolated from Crottin goat's cheese. Three groups of experiments were carried out in broth culture at different incubation temperatures: A (5 ° C), B (7 ° C) and C (9 ° C). A reference treatment in isothermal conditions was conducted for each group and four treatment with different periods at high temperature (25 ° C). The behavior was traced with OD measures. Isothermal treatments were analized by Gompertz model and two models of DMFit program. Lag time, specific growth rate and the maximum microbial density were determined. Parameters showed no clear overall preferences among the three models. At non-isothermal treatments the population increased proporcionally to period at 25 ºC for A and B groups. There was no difference among the different periods at high temperature for C group. The physiological cell state would be the cause of the differences in the number of generations observed among the groups. These findings underline the need for greater consumer education regarding safety food handling practices, in order to avoid potential health risks.
Keywords: Bacterial count. Cold temperature. *Escherichia coli.* Food microbiology. Mathematical model. Population growth.
Resumen
Comportamiento de *Escherichia coli* a bajas temperaturas en condiciones isotermales y no-isotermales. Se analizó el comportamiento de *Escherichia coli* a temperaturas subóptimas de crecimiento empleando cepas aisladas de queso de cabra tipo Crottin. Se realizaron tres grupos de experimentos en caldo de cultivo: A (5 ºC), B (7 ºC) y C (9 ºC). Para cada grupo se realizó un tratamiento de referencia en condiciones isotermales y cuatro tratamientos con diferentes períodos de abuso de temperatura a 25 ºC. El comportamiento se determinó por mediciones de DO. En condiciones isotermales se analizó con el modelo de Gompertz y dos modelos del programa DMfit. Se determinó la fase de latencia, la velocidad específica de crecimiento y la densidad máxima. No se observaron diferencias entre los tres modelos. En los tratamientos no-isotérmicos a 5 ºC y 7 ºC la población se incrementó en forma proporcional al tiempo de abuso a 25 ºC; a 9 ºC no hubo diferencias en el comportamiento para distintos períodos a temperatura elevada. Las diferencias podrían deberse al estado fisiológico de las células cuando se produce el incremento de la temperatura. Estos resultados enfatizan la necesidad de brindar mejor educación a la población sobre prácticas correctas de manipulación de los alimentos y evitar en consecuencia posibles riesgos para la salud.
Palabras clave: Baja temperatura. Crecimiento de la población. *Escherichia coli*. Microbiología de los alimentos. Modelo matemático. Recuento bacteriano.

Introduction In many developed countries the government's responsibility on food control is spread over an entanglement of organisms and jurisdictions, thus diminishing the effectiveness of many a control and leaving no guarantee regarding food's safety. Although there is a lack of research into local foodborne diseases, different media indicates that in the last years the amount of people affected by this cause has notoriously increased [2,3,4,23]. In the world Argentine is the country with the highest amount of people affected by haemolytic-uraemic syndrome (HUS), with *E. coli* (EHEC) the main etiologic agent [22, 29]. The prevention of pathogenic and spoilage bacteria in foods should be among the main concern of

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ISSN 1697-5529

manufactures and health authorities. Undesired microbial growth inside the processing environment can cause serious economical losses in the food industry. Spoilage microorganisms often outnumber pathogens and tend to be more resistant to harsh environmental conditions [24]. Altogether in the last decades consumers demand for high quality products with lighter processing and fewer preservatives has increased steadily. On most cases these products rely on refrigerated storage and distribution for their preservation from both microbial and quality retention standpoint [1]. Nonetheless the storage at refrigeration temperature can not always guarantee the desired safety and quality, as bacteria have many physiological adaptations to a wide variety of stressed environmental conditions [7, 30]. Besides, the application of sub-lethal food preservation techniques rather than lethal ones may contribute to the development and dissemination of antibiotic resistance among important food-borne pathogens [16]. In addition some retailers are not familiarized with the importance of maintaining the cold chain, and transitory temperature abuses could lead to more rapid growth of pathogenic bacteria than commonly expected [15, 19]. So, it is evident that risks associated with breaks in the cold chain are a reality [6]. Taking into consideration that bacteria are able to survive in refrigerated environment and that products are occasionally temperature abused, it is quite clear that an interruption in the cold chain would produce unknown or undesirable consequences. The economical and social consequences of the presence of microorganisms in foods depend not only on the species but mostly on the size of the populations. It is the number of microbial cells what ultimately determines whether or not the product will cause an outbreak or develop an off-flavor [12]. To elucidate the effect of environmental factors on the growth of different microorganisms it is necessary to accumulate sufficient numerical data. One of the simplest methods for data collection is the use of optical density (OD). Usually, mathematical models are used to analyze the microbial growth response. Modeling the growth and survival of pathogenic and spoilage microorganisms is a basic tool for the prediction of food safety and microbial deterioration of products in the food chain [18]. Different primary models are used to express changes in the concentration of microorganism over time using a limited number of kinetic parameters: lag time (λ), growth rate (μ) and maximum microbial density (γ_{max}). Altogether predictive models are an important tool in food safety risk assessment [17, 26]. In the current work we analyze the behavior of *E. coli* isolated from Crottin goat's cheese at different suboptimal growth temperatures, as well as the influence of different periods of temperature' abuse on the proliferation of these strains. Parallel the behavior of these strains was determined at high temperatures to compare it with reference ones.

Materials and methods Bacterial strains: two wild strains of *E. coli* isolated from Crottin goat cheese were used. This identity was determined by the API 20E Gram Negative Identification System (bioMerieux). The strains were maintained in Trytic Soy agar (Britania, Argentina). These were incubated without agitation at 25 ºC for 24 h in Trytic Soy broth (TSB, Britania, Argentina). A mixture containing equal quantity of cells of each strain was used as inoculum.

Experimental design: three groups of experiments were carried at different incubation temperatures: A group at 5 ºC; B group at 7 ºC; C group at 9 ºC. To follow the growth of each population the OD was measured with a Spectrophotometer (Metrolab ® 325) at 600 nm. For each group ten shaken flasks (150 rpm) with 125 ml of Trytic Soy broth previously equilibrated to the respective temperature were inoculated to obtain initial OD_{600} values of about 0.03. For each group two shaken flasks were used as reference at isothermal conditions. The other eight, after 24 h from the beginning of the experiment at their respective incubation temperatures, were taken to non-isothermal conditions. Two of them remained at 25 ºC for 1 hour, other 2 for two hours, other two for 3 hours and the final pair for 4 hours. Once each time limit was exceeded the respective pair of flasks was taken back to its incubation temperature. During each abusive temperature period and for approximately three hours after returning to low temperature the OD was measured on an hourly basis. Afterwards the OD was measured during different intervals till the beginning of the stationary phase; dilutions to OD < 0.3 were made at high cell densities. At random times the references cultures were analyzed to observe the cells length by Gram staining. The growth of the strains on isothermal conditions at 15, 20 y 35 ºC was examined parallel.

Data analysis: optical density values were converted to $\log_{10}$. The behavior of *E. coli* with time in all reference treatments was analyzed with modified Gompertz nonlinear model [31] using Origin 8.0 (OriginLab Corp.) and two models of DMFit program: DModel and Gompertz. The bacterial growth data collected were fitted to determined lag time (λ), specific growth rate (μ) and maximum microbial density (γ_{max}). To evaluate the reliability of the fitting the mean square error (MSE) was calculated. On the other hand the behavior of the strain in non-isothermal conditions was drawn with Origin 8.0**.** The behavior of μ with temperature was analyzed using square-root-type model [25]

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ISSN 1697-5529

Results

Isothermal conditions: the experimental OD values obtained were used to fit the primary mathematical models of growth on isothermal conditions. Growth kinetics in reference treatments of each *E. coli* experimental group is shown in Fig 1-3. The values of λ, μ and γ_{max} MSE in each reference experiment at the three temperatures are shown in Table I. At 5°C and 7 ºC the OD stabilized between days 8 and 6 respectively; at 9 ºC the high OD at day 3 made it impossible to continue with the experiment. The behavior at higher temperature is showed in Fig. 4"

Non-isothermal conditions: the behavior of *E. coli* under non-isothermal conditions is shown in Fig 5-7. Cultures were observed during 24 h after interrupting the incubation at low temperature (48 h after the beginning of the experiment) to analyze the number of generations produced. In A and B groups the population increased proportionally to the augment in the length of the high temperature period (Table II). In C group the population also increased, but there was no difference among the different periods of time at high temperature. In the experiments of A group the interruption of the low temperature incubation was made during the population's lag phase, in B group cells were at the beginning of log phase and in C at full log phase.

Table I. Kinetic parameter of *E. coli* reference treatment for A group (5 ºC), B group (7 ºC), and C group (9 ºC) with three mathematical models.

Group (Temperature)	Kinetic Parameters[1]	Models		
		Gompertz 1[2]	DModel[2]	Gompertz 2[3]
A (5 ºC)	$\lambda(h)$	35.81	27.51	39.73
	$\mu(h^{-1})$	0.009	0.008	0.009
	$y_{max}[\log\left(\frac{OD/ml}{OD_0/ml}\right)]$	1.35	1.58	1.46
	MSE	0.0012	0.0019	0.0012
B (7 ºC)	$\lambda(h)$	22.78	22.34	18.78
	$\mu(h^{-1})$	0.011	0.011	0.011
	$y_{max}[\log\left(\frac{OD/ml}{OD_0/ml}\right)]$	1.16	1.29	1.23
	MSE	0.0009	0.0019	0.0009
C (9 ºC)	$\lambda(h)$	16.21	16.15	16.46
	$\mu(h^{-1})$	0.037	0.036	0.037
	$y_{max}[\log\left(\frac{OD/ml}{OD_0/ml}\right)]$	2.04	2.52	2.51
	MSE	0.0007	0.0006	0.0006

[1] □: lag time, □□growth rate, y_{max}: maximum microbial density, MSE: mean square error
[2] DModel and Gompertz 1: models of DMFit program (http:/www.ifr.uk/safety:DMFit/)
[3] Gompertz 2: model modified (Van Impe et al., 1992)

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ISSN 1697-5529

Table II. Number of generations (n) occurred 24 h after break the incubation at low temperature in non-isothermal related to isothermal conditions for A, B and C groups.

Group	Break period at 25 ºC (h)			
	1	2	3	4
A (5º C)	1.24[(1)]	3.00	4.90	5.50
B (7º C)	1.60	2.58	4.26	5.10
C (9º C)	1.48	1.50	1.67	1.50

[(1)] the value is a quotient between the number of generations for each interruption time in non-isothermal conditions and under isothermal conditions (reference treatment).

Discussion

Temperature affects the rates of cell reactions, nutritional requirements and the biomass. It also has great impact on genetic expression. Cells living at low temperatures are not just slower in growth than those at environmental ones: they are also physiologically and genetically different. The adaptation to low temperature requires a coordinated, multifunctional response. In *E. coli*, independent mechanisms co-exists to ensure adaptation to low temperature growth. These are important to bacteria as they face changing environmental conditions [32].

OD measurements made possible real time tracing of bacterial population growth, as they provide a way to collect many data with relatively low effort. In spite of the problems associated with the use of turbidimetric data for modeling, this is an advantageous approach [20]. Among other things it may be used for the reliable estimation of μ_{max} [11]. Spoilage bacteria become an issue in foods when high levels growth takes place, so absorbance techniques will most often be of great practical importance [10]. Growth of food poisoning bacteria in foods generally follows a similar pattern to that in laboratory media [8].

Isothermal conditions: in general, as the incubation temperature increased a shorter lag time duration and a faster growth rate were observed. Temperature influences the participation of different biochemical routes. Jones *et al*. [13] did not found a lag phase at 7 ºC with a wild strain of *E. coli*. However that lag phase did appear in our results at the same incubation temperature. This is probably due to the inoculation of the flasks with cells which were not cold-adapted, as lag depends on the physiological state of the cells. Probably the temperature affects not only the growth rate, but also the growth yield. Jones *et al*. [13] determined that at 7, 8, 9 and 10 ºC the absorbance values of *E. coli* increased with time, but at 7 ºC the maximum absorbance value was lower than the values attained at highest incubation temperature. These results are concordant with ours. These authors also found that when cultures were incubated at 7 ºC cells' optical absorbance and length increased. In the Gram staining controls made with cultures at random times in isothermal conditions we could see a large number of elongated cells. This took place in all three incubation temperatures. Cell elongation when environmental conditions approach growth's limits may lead to a misunderstanding of the health risks associated with the number of some pathogens recovered from foods.

Fitted curves in Figures 1-3 show no clear overall preferences to the eye. The mean square error (MSE), which evaluates the reliability of the fitting, was calculated [21]. Table I shows that the MSE of all curves was very similar. The DMFit program (an Excell add-in) proved an advantageous tool to fit experimental data set and to describe the bacteria growth kinetics. The behavior of these strains of *E. coli* at higher incubation temperature showed similar patterns to another strains (Figure 4) [27]. These results confirm that OD measurements are an important tool to follow bacterial growth. The OD measurements would reflect the potential number of bacteria to which consumers would be exposed.

Non-isothermal conditions*:* the physiological cell state would be the cause of the differences observed among the groups in the number of generations. These results indicate that a cut in the cold chain produced while bacteria are between 5 and 7 °C

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would lead to a shorter generation time, proportional to the lapse of high temperature exposition. On the other hand, if cells are in log phase and at higher temperatures (≥9°C) although an increase in the number of generations would occur, there would be no variations in this value for the different periods of temperature rise (from 1 to 4 h).

Rajkowski and Marmer [19] found that growth kinetics at fluctuating temperatures approximated the higher temperature more closely than the midpoint. Bacteria can quickly respond to changing temperatures by regulating gene expression through both RpoS-dependent and RpoS-independent thermoregulatory mechanisms [32]. In contaminated foods at refrigeration temperature the possibility of rapid septation of filamentous cells would rise upon warming. Likewise, cells in different physiological state may respond differently to other stresses during warming of chilled foods. Jones *et al*. found that health risk from mesophilic pathogens as a result of exposure of chilled foods to abusive temperatures may differ in foods stored at less than 2 ºC with those above 2 ºC [14]. Transitory temperature abuse could lead to more rapid growth of some pathogens than expected.

An understanding of how *E. coli* responds to temperature changes is important in food industry where prevention of bacterial contamination is imperative. If optimal temperatures during storage and transportation of chilled foods are not ensured, its microbial alteration can be greatly accelerated. In Argentine the elevated estival temperatures often lead to breaks in the cold chain, produced by blackouts which are a usual consequence of the current crisis [5]. In the light of these circumstances the adoption of preventive measures becomes a crucial factor to minimize health risks. It has been shown how changes in food safety education would improve the public health [9, 28]. So educating people about the importance of food quality and safety is considered one of the most effective ways to reach that objective.

Works like this provides relevant scientific information and would assist the food industry to consider the need to control the temperature more efficiently. Also underline the need for greater consumer education regarding safe food handling practices in order to avoid health risks derived from microbial food spoilage.

Acknowledgements. This work was partially financed by Secretaría de Ciencia y Técnica, Córdoba, Argentina. We are grateful to C.E. Budde Jr. for his technical assistance.

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Figures:

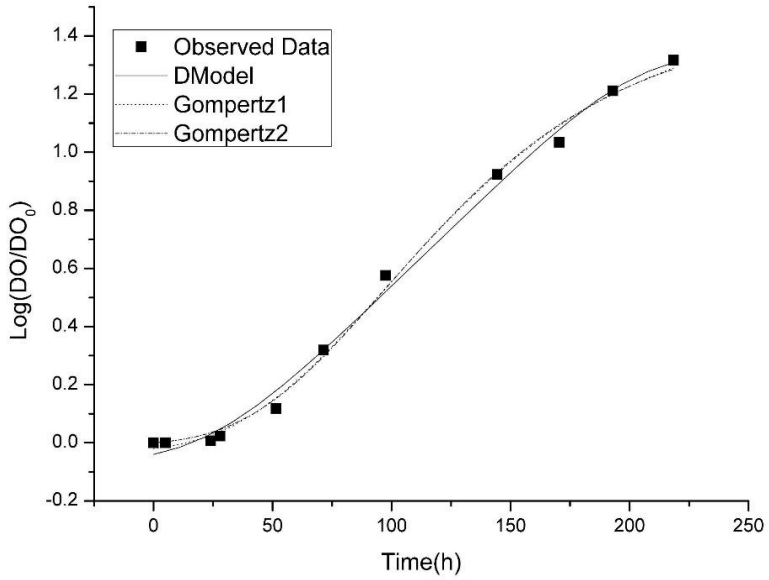

Fig 1. Observed data (■) and predicted curves (Gompertz 1 ·····, DModel ——, Gompertz 2 –·–·–·) in reference treatment for *E. coli* at 5ºC.

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ISSN 1697-5529

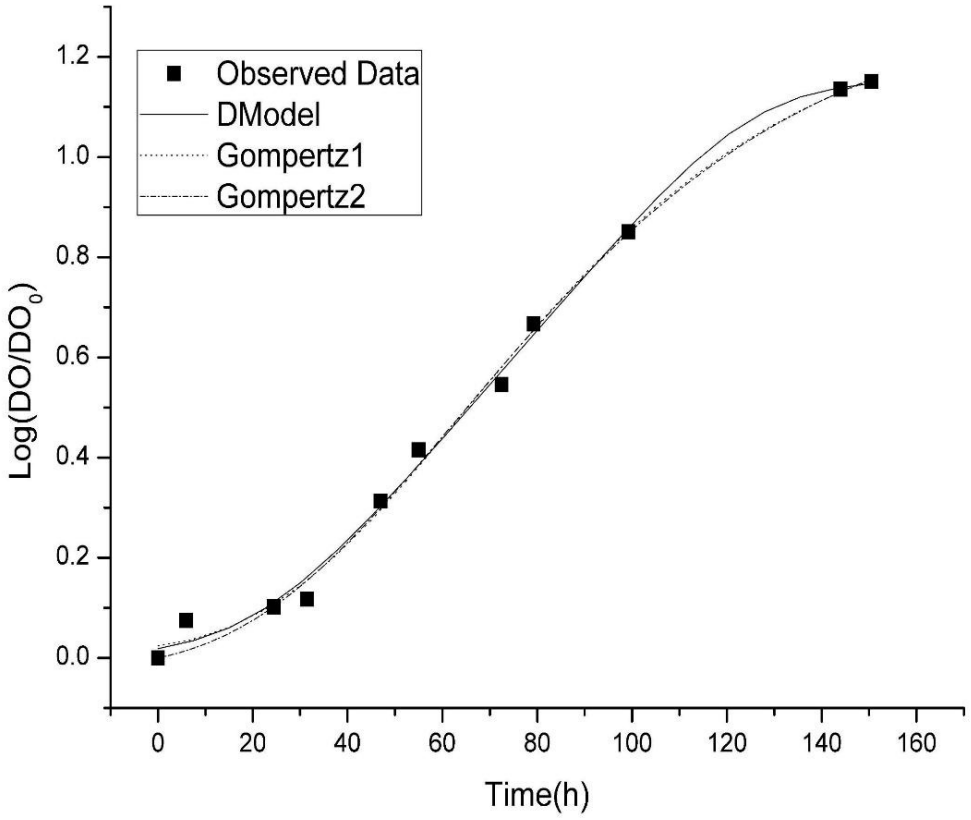

Fig 2. Observed data (■) and predicted curves (Gompertz 1 ·····, DModel ——, Gompertz 2 –·–·–·) in reference treatment for *E. coli* at 7ºC.

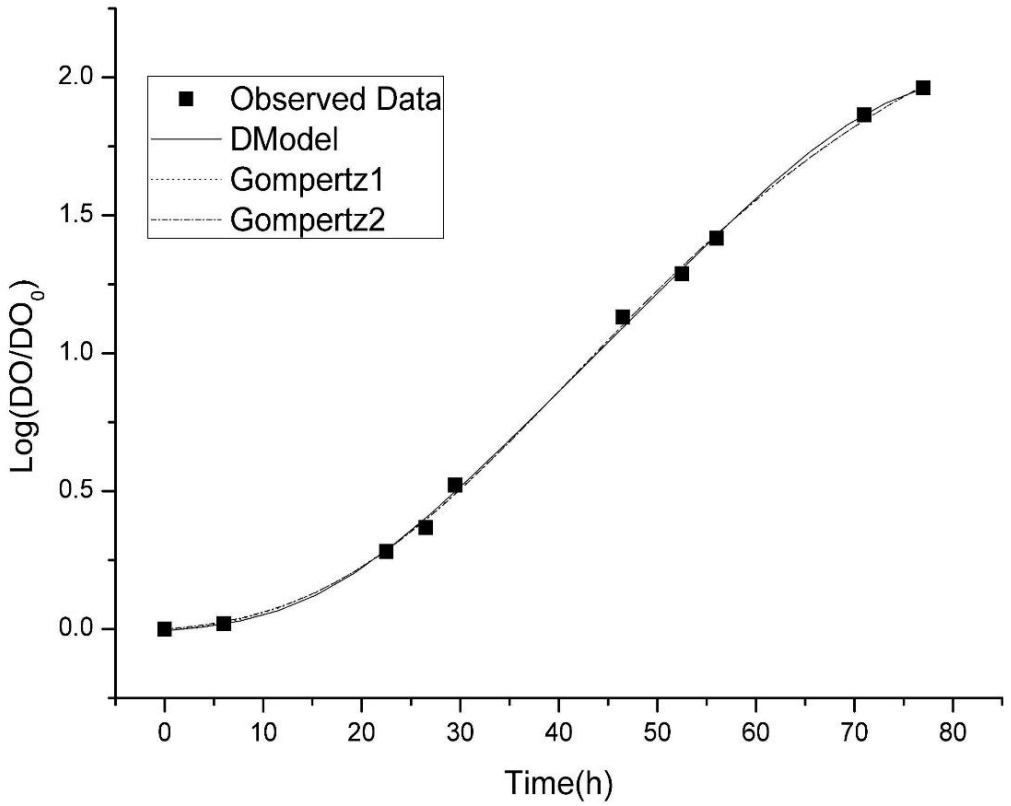

Fig 3. Observed data (■) and predicted curves (Gompertz 1 ·····, DModel ——, Gompertz 2 –·–·–·) in reference treatment for *E. coli* at 9ºC

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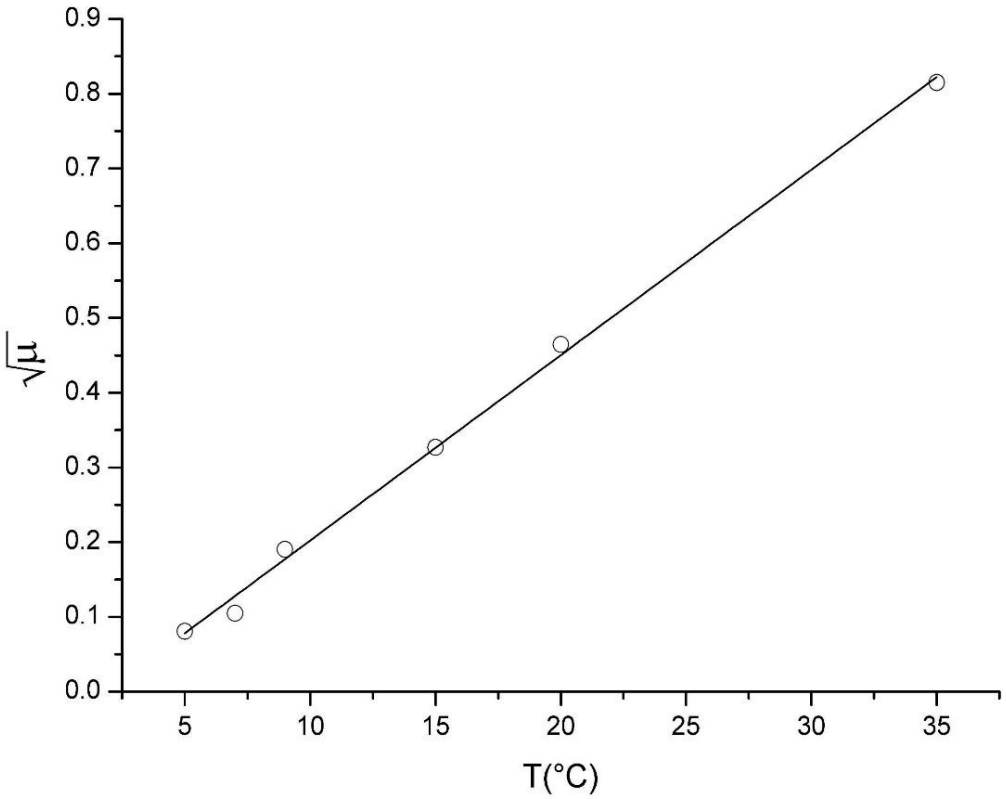

Fig 4. Square root of rate μ (h^{-1}) as a function of temperature T (ºC).

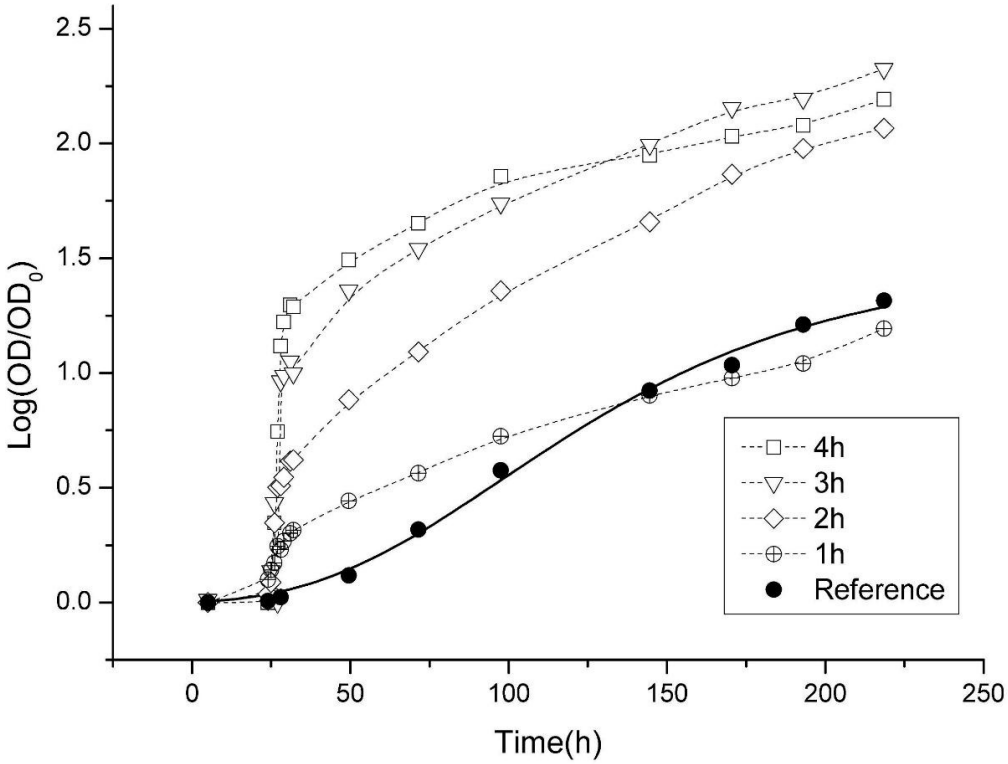

Fig 5. Effect of break in the cold chain at 5 ºC for E. coli. Reference treatment (black circle); break during 1 h at 25 ºC (circled plus); break during 2 h at 25 ºC (diamond), break during 3 h at 25 ºC (triangle down),break during 4 h at 25 ºC (square). Dashed lines are only to guide the eye

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ISSN 1697-5529

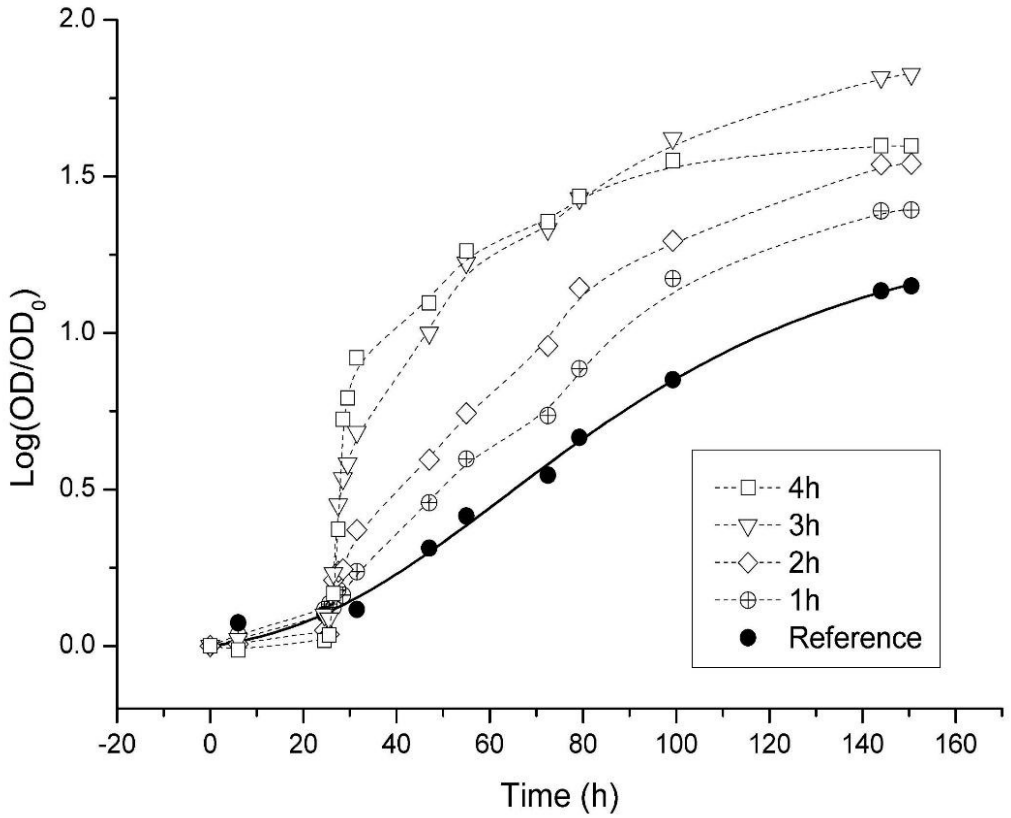

Fig 6. Effect of break in the cold chain at 7 ºC for E. coli. Reference treatment (black circle); break during 1 h at 25 ºC (circled plus); break during 2 h at 25 ºC (diamond), break during 3 h at 25 ºC (triangle down),break during 4 h at 25 ºC (square). Dashed lines are only to guide the eye.

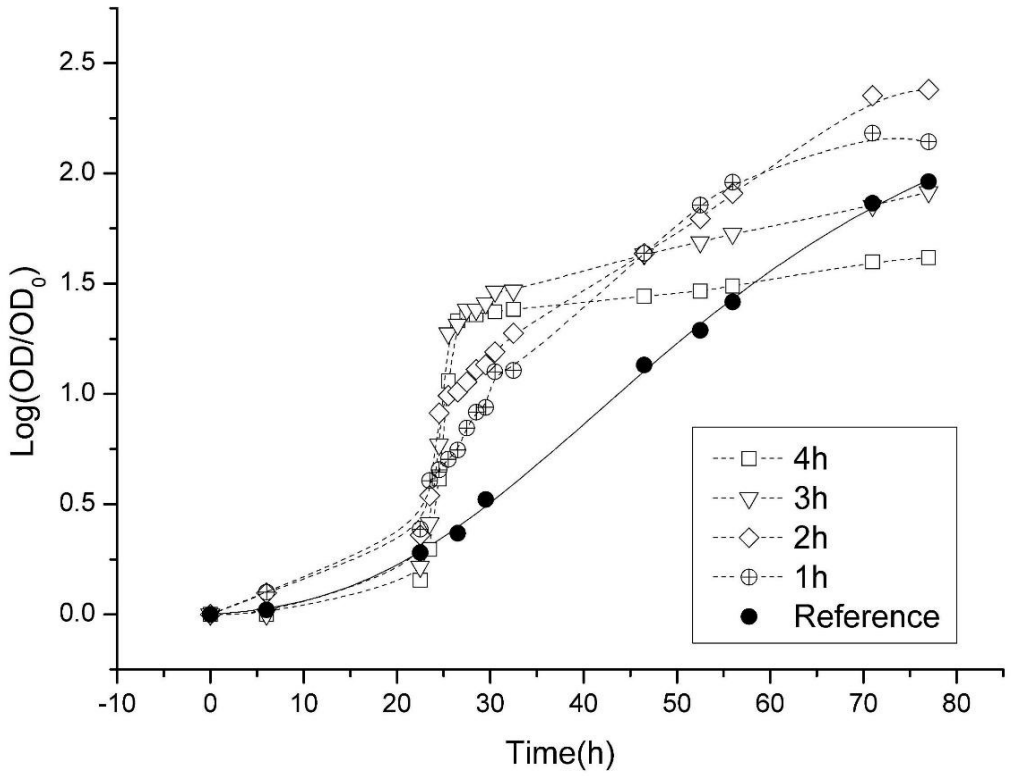

Fig 7. Effect of break in the cold chain at 9 ºC for E. coli. Reference treatment (black circle); break during 1 h at 25 ºC (circled plus); break during 2 h at 25 ºC (diamond), break during 3 h at 25 ºC (triangle down),break during 4 h at 25 ºC (square). Dashed lines are only to guide the eye.