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Studying the growth boundary and subsequent time to growth of pathogenic *Escherichia coli* serotypes by turbidity measurements

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ABSTRACT

The presence of *Escherichia coli* in contaminated food products is commonly attributed to faecal contamination when they are improperly handled and/or when inactivation treatments fail. Adaptation of *E. coli* at low pH and a_w levels can vary at different temperatures depending on the serotype, thus more detailed studies are needed. In this work, a screening to assess the growth of four pathogenic serotypes of *E. coli* (O55:H6; O59:H21; O158:H23 and O157:H7) was performed. Subsequently, boundary models were elaborated with the fastest serotype selected at different temperatures (8, 12 and 16 °C), and inoculum levels (2, 3 and 4 log cfu/mL) as function of pH (7.00–5.00) and a_w (0.999–0.960). Finally, the growth kinetics of *E. coli* was described in the conditions that allowed growth. Results obtained showed that the serotypes O157:H7 and O59:H21 did not grow at more stringent conditions (8 °C; pH 5.50), while the *E. coli* O158:H23 was the best adapted, resulting in faster growth. The logistic regression models presented a good adjustment to data observed since more than 96.7% of cases were correctly classified. The growth interface was shifted to more limited conditions as the inoculum size was higher. Detection times (t_d, h) and their variability were higher at low levels of the environmental factors studied. This work provides insight on the growth kinetics of *E. coli* at various environmental conditions.

1. Introduction

Entero-pathogenic *Escherichia coli* comprise different serotypes that can be present in contaminated foods causing several human illnesses. Its pathogenicity primarily depends on the virulence genes acquired. Most of these serotypes are capable to produce Shiga-like toxins and/or other heat labile or heat stable toxins that can potentially cause diarrhogenic diseases in humans. Entero-haemorrhagic *E. coli* can also cause haemolytic uraemic syndrome which has a relatively high mortality rate (5–15%) (Chu and Hemphill, 2004). Besides, some serotypes of entero-toxigenic *E. coli* can also produce a cytotoxin to Vero cells (VTEC *E. coli*).

Infections caused by VTEC serotypes are of low prevalence (overall EU total of 1.2 cases per 100,000 people in the EU) (EFSA, 2006); but the high infectivity and seriousness of disease justifies the consideration of these microorganisms as important foodborne pathogens. Outbreaks attributed to *E. coli* serotypes within the EU increased by 38.3% in 2007 compared to 2006 being the largest number of human cases (39.4%) originated from outbreaks in catering services or restaurants (EFSA, 2009).

Generally, *E. coli* can be present in foods from animal origin, such as pork, beef or poultry products (Carney et al., 2006), tap water (Solomon et al., 2002) or in fresh-cut leafy greens vegetables such as cabbage, lettuce or spinach (Bharathi et al., 2001). They can enter the food chain through cross-contamination or recontamination phenomena (Pérez-Rodríguez et al., 2007) or through the irrigation with contaminated water, which may result in the internalization of certain *E. coli* serotypes in vegetables (Sivapalasingam et al., 2004).

Also, it has been reported the acid resistance and subsequent adaptation of several *E. coli* serotypes as a result of their presence in low-pH foods and in the runoff fluids that remain in meat industries after applying disinfection treatments with organic acids in meat carcasses (Samelis and Sofos, 2003; Stopforth et al., 2007).

Particularly, *E. coli* O157:H7 is of great interest due to the severity of the illnesses caused and its low infective dose, around 100 cells (Strachan et al., 2005). However, other non-O157 serotypes have been associated to human infections (Blanco et al., 2004) through the ingestion of risk food products, such as fermented and minced meats or raw milk (Scavia et al., 2005). It is recognized that these outbreaks could be under-reported because surveillance is mainly based on laboratory methods specific for *E. coli* O157 (Possé et al., 2007). Besides, characterization, survival and growth behaviour of non-O157 serotypes in foods have not been yet sufficiently studied.

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A comparison between different serotypes in order to know more accurately the survival and growth capacity of *E. coli* at optimal and limiting environmental conditions simulating food matrices could be of high interest.

In this sense, predictive models have been defined as a powerful tool to describe microbial behaviour in foods. Specifically, boundary models can be used to determine the chance of microorganisms growing at specific levels of environmental factors. Therefore, these models are greatly relevant in the safety of foods which can be contaminated with food pathogens (Vermeulen et al., 2007). Traditionally, the output of these models, i.e. probability of growth (P), has been set up in fail-dangerous cut off points (0.5) that could lead to unacceptable predictions for food industries in the case of pathogens (50% chance of growth). Salter et al. (2000) stated that the level of probability can be set depending on the level of safety required. They concluded that safer cut off point probability levels would produce a shift in the growth/no growth boundary to limiting levels of environmental factors (temperature and a_w). Therefore, setting low levels of P are necessary in order to provide more accurate growth interfaces.

Among many other factors, the position of the growth/no growth boundary is affected by the inoculum level as is shown in some studies (Masana and Baranyi, 2000; Koutsoumanis and Sofos, 2005). Skandamis et al. (2007) found that the interface was shifted to low levels of environmental conditions when the inoculum level was high. Similar results were published by Bidlas et al. (2008), suggesting that the inoculum effect can be modelled independently, and the growth/no growth boundary is only influenced by the inoculum size. However, the potential growth of low inoculum levels at limiting conditions can be associated to different sources of variability attributed to their physiological state, molecular cell mechanisms that induce growth, or composition of the media. Unfavourable conditions to grow induced by low levels of environmental factors such as temperature, pH or water activity increase the variability of cells to start growing.

The main purpose of this work was to study the behaviour of *E. coli* serotypes in the growth/no growth domain as a function of four different factors: inoculum level, temperature, pH and a_w. A screening to assess the growth of four *E. coli* serotypes (O55:H6; O59:H21; O158:H23 and O157:H7) was performed at different temperatures, pH and inoculum levels by turbidimetric measurements. Afterwards, the fastest serotype was selected to perform boundary models against pH and a_w at different temperatures and inoculum levels. Finally the detection time (t_d) was calculated in the conditions in which growth was observed.

2. Material and methods

2.1. Serotypes and inoculum

In a preliminary phase, a screening of four different *E. coli* serotypes was performed in order to select the fastest one to elaborate the boundary models. All serotypes have been isolated from contaminated foods and can cause diverse human diseases:

- *E. coli* O55:H6 (NCTC 8959): has been isolated from contaminated foods, presenting virulence properties such as plasmids and genes for the pilin subunit of the bundle-forming pilus which are related to the local adherence to live tissues and DNA hybridization (Pelayo et al., 1999).
- *E. coli* O59:H21 (ATCC 10536): is an entero-toxigenic serotype that has been associated to human disease (Arthur et al., 2002).
- *E. coli* O158:H23 (NCTC 10974): has been catalogued as an entero-pathogenic serotype associated to outbreaks of infantile enteritis (Rowe et al., 1974).
- *E. coli* O157:H7 (ATCC 35150): is a VerocytoToxigenic serotype that has been involved in several food outbreaks causing 76 million cases of human illness and up to 5,000 deaths annually in the US (Frenzen et al., 2005).

The serotypes were obtained from the Spanish Type Culture Collection (CECT, Burjassot, Valencia) and rehydrated as indicated by the manufacturer's instructions. All serotypes showed a typical growth on McConkey-Sorbitol and on Tryptone Bile X-Glucuronide Medium, TBX agar (Oxoid, UK).

Three days before the experiment, stock cultures of each serotype were transferred to a tube containing 10 mL of Tryptone Soya Broth (TSB, Oxoid ltd., Basingstoke, Hampshire, UK) and incubated at 37 °C for 24 h. From this, 1 mL was sub-cultured into a tube containing 10 mL of TSB and incubated at 37 °C for 24 h. One more time, *E. coli* strains were sub-cultured in a 50 mL flask until the early stationary phase was reached (18 h).

2.2. Experimental design

2.2.1. Screening of the serotypes

The screening of *E. coli* serotypes was performed in modified culture media at three temperatures (8, 12, 16 °C), two levels of pH (7.00 and 5.50), two inoculum levels (2 and 4 log cfu/mL) at a fixed value of a_w (0.999). Therefore, 12 different environmental conditions were selected for each serotype, and three replicates were performed per condition tested.

2.2.2. Boundary models

A full factorial design was followed for the elaboration of the boundary models with nine levels of pH (7.00, 6.75, 6.50, 6.25, 6.00, 5.75, 5.50, 5.25 and 5.00) and five levels of a_w (0.999, 0.990, 0.980, 0.970 and 0.960). Each model was performed at three different temperatures (8, 12, 16 °C) and inoculum levels (2, 3 and 4 log cfu/mL). Thus, 45 conditions of pH and a_w were studied per temperature and inoculum level, obtaining finally nine boundary models.

2.3. Media preparation

Water activity levels were set by adding the necessary quantities of sodium chloride (Panreac, 131659) to Tryptone Soya Broth (TSB, Oxoid, UK) 100 mL flasks. The sodium chloride percentage was calculated considering the salt content of the initial TSB broth (5.0 g/L). a_w was subsequently measured with Aqualab model 3TE (Decagon Devices, Inc., Pullman, Washington, USA).

Adjustment of pH was made with a 1 M HCl solution (Panreac, 181021), and 1 M NaOH solution (Panreac, 181691) when necessary, and pH values were measured with a pH/mv-meter digit 501 (Crison, Barcelona, Spain). Once modified, all media were filter-sterilized through 0.22 μm sterile filters (Millipore, Madrid Spain) and they were kept in refrigeration until inoculation. Before inoculation, a_w and pH values were checked.

2.4. Inoculation procedure and assessment of growth

When the early stationary phase was reached by the inocula (~18 h), the 50 mL flasks were decimal diluted in 9 mL tubes of physiologic saline solution at 0.85% until obtaining a concentration of 5×10^2; 5×10^3 and 5×10^4 cfu/mL. Afterwards, a number of microtiter wells per condition (3 wells for screening, and 8 wells for growth/no growth models development) were filled up with 200 μL of the modified media, and inoculated with 50 μL of the diluted cultures, reaching the desired concentration; i.e. 10^2, 10^3 or 10^4 cfu/mL per well. In addition, 2 wells per condition studied