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Variability in growth/no growth boundaries of 188 different *Escherichia coli* strains reveals that approximately 75 % have a higher growth probability under low pH conditions than *E. coli* O157:H7 strain ATCC 43888

L.U. Haberbeck [a, b], R.C. Oliveira [c], B. Vivijs [a], T. Wenseleers [c], A. Aertsen [a], C. Michiels [a], A.H. Geeraerd [b, *]

[a] Laboratory of Food Microbiology, KU Leuven, Kasteelpark Arenberg 22, B-3001 Leuven, Belgium
[b] MEBIOS–Division of Mechatronics, Biostatistics and Sensors, Department of Biosystems (BIOSYST), KU Leuven, W. de Croylaan 42, B-3001 Leuven, Belgium
[c] Laboratory of Socioecology and Social Evolution, Zoological Institute, KU Leuven, Naamsestraat 59–Box 2466, 3000 Leuven, Belgium

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A B S T R A C T

This study investigated the variation in growth/no growth boundaries of 188 *Escherichia coli* strains. Experiments were conducted in Luria–Bertani media under 36 combinations of lactic acid (LA) (0 and 25 mM), pH (3.8, 3.9, 4.0, 4.1, 4.2 and 4.3 for 0 mM LA and 4.3, 4.4, 4.5, 4.6, 4.7 and 4.8 for 25 mM LA) and temperature (20, 25 and 30 °C). After 3 days of incubation, growth was monitored through optical density measurements. For each strain, a so-called *purposeful selection approach* was used to fit a logistic regression model that adequately predicted the likelihood for growth. Further, to assess the growth/no growth variability for all the strains at once, a generalized linear mixed model was fitted to the data. Strain was fitted as a fixed factor and replicate as a random blocking factor. *E. coli* O157:H7 strain ATCC 43888 was used as reference strain allowing a comparison with the other strains. Out of the 188 strains tested, 140 strains (~75%) presented a significantly higher probability of growth under low pH conditions than the O157:H7 strain ATCC 43888, whereas 20 strains (~11%) showed a significantly lower probability of growth under high pH conditions.

1. Introduction

Escherichia coli is naturally present in the gastrointestinal tract of humans and other animals and, in general, is not harmful to the host. Nonetheless, certain *E. coli* strains have acquired specific virulence genes which induce the ability to cause a large number of diseases (Ahmed et al., 2008). Two factors allow *E. coli* to acquire and lose those virulence genes at a relatively high frequency: the high plasticity of the genome and the fact that most of the virulence genes are encoded in mobile elements such as plasmids, phages or transposons (Kuhnert et al., 2000). Gene transfer occurs in many environments leading to strains with new combinations of virulence genes that might emerge in the future (Kaper et al., 2004; Kelly et al., 2009).

E. coli O157:H7 is the major food-borne pathogen linked to outbreaks related to ground beef products (Doyle, 1991). The main reservoir of this bacteria is known to be the bovine gastrointestinal tract (Price et al., 2004). However, many outbreaks were also linked to other foods types including acid foods like mayonnaise (Weagant et al., 1994), apple cider (Zhao et al., 1993) and yogurt (Morgan et al., 1993). The mechanisms used by *E. coli* to survive in acidic environments involve an increase of internal pH and a change in both transmembrane electrical potential and metabolic activity (Foster, 2004).

Knowledge of microorganism growth limits under different environmental conditions allows for a better quality and safety management of foods (McMeekin et al., 2000). Models used to define the growth limits are known as growth/no growth interface, growth boundary or growth limit models (Ross and Dalgaard, 2004). Growth/no growth models can predict suitable combinations of hurdles making microbial growth highly unlikely (Masana and Baranyi, 2000). Different methodologies to observe *E. coli* growth boundaries under different combinations of environmental

* Corresponding author. Tel.: +32 16 32 05 91.
E-mail addresses: leticia.haberbeck@kuleuven.be (L.U. Haberbeck), Annemie.Geeraerd@kuleuven.be (A.H. Geeraerd).

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factors can be found in literature (McKellar and Lu, 2001; Presser et al., 1998; Salter et al., 2000; Skandamis et al., 2007; Valero et al., 2010). Most studies consider either a limited number of strains or a mix of strains. Valero et al. (2010) performed experiments using four different *E. coli* serotypes at different temperature, pH and inoculum levels. McKellar and Lu (2001) observed the growth boundaries for a mix of five *E. coli* O157:H7 strains according to temperature, pH and concentration of acetic acid, salt and sucrose, in a system mimicking a mayonnaise sauce. Skandamis et al. (2007) modeled the growth boundaries of nonadapted and acid-adapted *E. coli* O157:H7 (mixture of 4 strains) influenced by pH, NaCl concentration and temperature.

Since variability between strains may have an important impact on the accuracy of risk assessment outcomes (Lianou and Koutsoumanis, 2013), information about the variability in phenotypic responses among strains of the same species under different environmental conditions is crucial (Nauta and Dufrenne, 1999). The variability between strains of the same species is discussed in the literature through several biological observations, like variability in heat resistance, biofilm formation, growth behavior and acid resistance. So far, however, a limited amount of data regarding the variability in growth/no growth boundaries among different strains is available in literature (Lianou and Koutsoumanis, 2013). Experiments on the growth kinetics of 17 *E. coli* O157:H7 strains in brain heart infusion adjusted to pH 5.3 with lactic acid demonstrated that the lag phase could vary from 13.7 to 55.6 h (Whiting and Golden, 2002). Heat inactivation at 60 °C in a simulated beef gravy medium of five *E. coli* serotypes (O157:H7 and non-O157:H7) from clinical and food isolates showed a significant difference in survival curves (Juneja and Marks, 2005).

Mixed-effects modeling is a statistical approach to model hierarchical data structures by clustering observations into groups that may arise from repeated measurements on the same strains or individuals (Schielzeth and Nakagawa, 2013). Despite being a state of the art statistical approach, mixed-effects models are not frequently used in predictive microbiology, exceptions being Juneja and Marks (2005), Krulikosjá et al. (2011), Mand et al. (2013) and Shorten et al. (2004). In mixed-effects models two types of effects are considered to model the data: the *fixed effects*, whose levels are experimentally determined such as temperature, pH, selected strain, and the *random effects*, whose levels are sampled from a large population and are due to biological variability (Bolker et al., 2009). Another definition for fixed and random effects is that fixed effects are related to unknown parameters to be estimated from data and random effects govern the variance-covariance structure of the independent variable (Crawley, 2007). For example, random effects include variation among individuals when multiple responses are measured per individual, region, genotype or species. Fixed effects are indeed the variables included in the statistical model, but random effects are strictly speaking not variables but unobserved random variation (Bates, 2010). Generalized linear mixed model (GLMM) combines the properties of two statistical approaches: mixed-effects models and generalized linear models. Generalized linear models (GLM) are a generalization of the ordinary linear regression but allowing a linear model to be related to response variables that are not normally distributed through the use of an appropriate link function, e.g. the logit link $log(p/1-p)$ in the case of logistic regression, where a binomially distributed dependent variable, with probability of occurrence p, is related to one or more continuous covariates.

The objectives of this study were (a) to observe the growth/no growth interfaces of 188 *E. coli* strains isolated from different sources, (b) to model the growth/no growth interfaces of each *E. coli* strain with a logistic regression technique, (c) to model the variation among the strains and assess the level of variability among them through the use of GLMM, and (d) to assess whether highly resistant *E. coli* strains can be isolated from the environment.

2. Material and methods

2.1. Bacterial strains and inoculum preparation

The collection of *E. coli* strains comprised 18 avian pathogenic *E. coli* (APEC) strains obtained from Prof. B. Goddeeris (KU Leuven, Belgium), 20 cytotoxic necrotizing factor (CNF)-producing (type 1 and 2), 8 necrotoxic (NETEC II), 14 enteropathogenic (EPEC) and 8 enterotoxigenic (ETEC) strains from Prof. J. Mainil (Ulg, Liège, Belgium), 20 strains from the *E. coli* Reference Collection (ECOR) (http://www.shigatox.net/new/reference-strains/ecor.html), 97 strains from the Laboratory of Food Microbiology (KU Leuven, Belgium) collection isolated from diverse environment sources (coded as EC or BV followed by a number), *E. coli* O55:H5 (ATCC 12014), O29 (ATCC 43892), O157:H7 (ATCC 43888) and MG1655. Stock cultures were maintained at −80 °C in Luria–Bertani media (LB) with 25% vol/vol glycerol. Active cultures of each strain were obtained by streaking a loopful of the frozen stock cultures into stock plates (LB agar plates) followed by incubation for 24 h at 37 °C. Stock plates were kept at 4 °C and redone every 2 weeks. Pre-inoculum cultures were obtained by picking one single colony from the stock plates and incubating it using LB media (pH 7) into microtiter plates overnight at 37 °C.

2.2. Experimental design

Growth/no growth interfaces were evaluated with respect to temperature (20, 25 and 30 °C), pH (from 3.8 to 4.8) and presence of LA (0 and 25 mM). The LB media pH with 25 mM of LA was adjusted with NaOH 1 M in six levels: from 4.3 to 4.8 with increments of 0.1 pH units. The LB media pH without LA (0 mM) was adjusted with HCl 37% also in six levels: from 3.8 to 4.3 with increments of 0.1 pH units. For all conditions pH was aseptically adjusted using a digital pH meter (Hanna, HI9125) after autoclaving. The HCL and NaOH solutions were filter sterilized before use. In total, 36 conditions per strains and four biological replicates (four different single cell colonies from the activated cultures in LB agar plates) per condition were tested. For half of the strains, the four biological replicates were extended to eight biological replicates at 20 °C, 0 mM LA and pH from 3.8 to 4.0. For *E. coli* O157:H7 strain ATCC 43888, eight biological replicates for all conditions and 12 biological replicates at 20 °C, 0 mM LA and pH from 3.8 to 4.0 were tested.

2.3. Growth/no growth experiments

Pre-inoculum cultures were diluted in LB media with adjusted pH, added to microtiter plates, to reach an initial inoculum of approximately 10^5 CFU/ml. All microtiter plates were sealed with a special cover (Enzyscreen, http://www.enzyscreen.com) that limits the evaporation and ensures equal oxygen conditions in all wells. Afterward the sealed microtiter plates were placed into clamp systems (Enzyscreen, http://www.enzyscreen.com) mounted on orbital shakers and incubated at the respective temperature. After three days of incubation the optical density (OD) was measured at 600 nm (Multiskan RC, Thermo Labsystems). Growth was considered to have occurred when the OD was higher than 0.150. This value was chosen during preliminary experiments by generating a growth curve at pH 7 and 37 °C for *E. coli* MG1655 correlating OD measurements and number of cells by plate counting. No subsequent plating to check for contamination was done. Instead, one of the microtiter plates' wells was not inoculated (blank) and if it did

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