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Effect of cell immobilization on the growth dynamics of *Salmonella*

Typhimurium and *Escherichia coli* at

suboptimal temperatures

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ABSTRACT

Predictive microbiology has recently acknowledged the impact of the solid(like) food structure on microbial behavior. The presence of this solid(like) structure causes microorganisms to grow as colonies and no longer planktonically as in liquid. In this paper, the growth dynamics of *Salmonella* Typhimurium and *Escherichia coli* were studied as a function of temperature, considering different growth morphologies, i.e., (i) planktonic cells, (ii) immersed colonies and (iii) surface colonies. For all three growth morphologies, both microorganisms were grown in petri dishes. While *E. coli* was grown under optimal pH and water activity (a_w), for *S. Typhimurium* pH and a_w were adapted to 5.5 and 0.990. In order to mimic a solid(like) environment, 5% (w/v) gelatin was added. All petri dishes were incubated under static conditions at temperatures in the range [8.0°C-22.0°C]. Cell density was determined via viable plate counting. This work demonstrates that the growth morphology (planktonic vs. colony) has a negligible effect on the growth dynamics as a function of temperature. The observation of almost equal growth rates for planktonic cultures and colonies is in contrast to literature where, mostly, a difference is observed, i.e., $\mu_{\text{planktonic cells}} \geq \mu_{\text{immersed colonies}} \geq \mu_{\text{surface colonies}}$. This difference might be due to shaking of the liquid culture in these studies, which results in a nutrient and oxygen rich environment, in contrast to the diffusion-limited gel system. Experiments also indicate that lag phases for solid(like) systems are similar to those for the planktonic cultures, as can be found in literature for similar growth conditions. Considering the maximum cell density, no clear trend was deducted for either of the microorganisms.

This study indicates that the growth parameters in the suboptimal temperature range do not depend on the growth morphology. For the considered experimental conditions, models previously developed for liquid environments can be used for solid(like) systems.

Keywords: suboptimal temperature; growth morphology; surface colony; immersed colony; planktonic cells; solid food structure

1. INTRODUCTION

At the end of the 20th century, predictive microbiology acknowledged the impact of solid(like) food structure on microbial behavior, after focusing for many decades on microbial dynamics in liquid systems. In liquid media, microorganisms grow planktonically or as freely-dispersed cells. Convective transport of nutrients, oxygen and metabolites leads to a locally uniform environment (Dens and Van Impe, 2001; McMeekin et al., 2002). Solid(like) food structure leads to the immobilization of microorganisms as isolated cells. When multiplying, they are constrained to grow as colonies (Brocklehurst et al., 1997). Compared to liquid systems, where convection is the main transport mechanism, the diffusion-based transport in a solid(like) food structure is slow. This results in a limited removal of toxic metabolites that are produced during colony growth, like acids. Additionally, the diffusion limitations influence the delivery of nutrients and oxygen to the growing colonies. As a consequence, concentration gradients of nutrients (Antwi et al., 2006), metabolites, pH (Malakar et al., 2000) and oxygen (Wimpenny and Coombs, 1983) will develop around the colony. A solid environment causes an additional stress to colonies, ultimately resulting in slower growth and a confinement of the growth domain compared to the liquid systems (Noriega et al., 2010b; Theys et al., 2008; Wilson et al., 2002). Additionally, the solid matrix causes changes in metabolism, cell development, morphology, membrane permeability, surface tension, osmotic pressure, and affects cell variability and physiological state (Dervakos and Webb, 1991; Meldrum et al., 2003; Noriega et al., 2013; Wilson et al., 2002).

The initial cell density or inoculum level also plays an important role in colony size, distribution and interactions. Low inoculum levels ($<10^3$ CFU/mL) lead to very big and well separated colonies, which are less active or even have dead cells in the colony center. For these low densities, only intra-colony

interactions occur since the space between two neighboring colonies is large. For high initial cell densities ($>10^3$ CFU/mL), colonies are small, grow very close to each other and thus inter-colony interactions become important (Malakar et al., 2002, 2003).

When studying the effect of a solid(like) environment on microbial growth dynamics, another important aspect to be considered is the colony location, i.e., colonies can either be immersed within the system or grow on the surface. Diffusion limitations have been reported to be greater at the solid surface than within an enveloping gel. This was confirmed by Peters et al. (1987), Robinson et al. (1991) and Wimpenny and Coombs (1983), who measured the depletion of oxygen and accumulation of protons immediately beneath the colony and extending into the substrate. Measurements of colony growth on gelatin surfaces resulted in a decreased growth rate as compared to that in broth or within gel systems. Comparisons of the growth rates of *S. Typhimurium*, for different salt and sucrose levels, followed the order: planktonic cells $\geq$ immersed colonies $\geq$ surface colonies (Brocklehurst et al., 1997). Other studies have confirmed that microbial growth on or within a gel system is slower than growth in liquid media (Theys et al., 2008; Wilson et al., 2002). However, Malakar et al. (2003) observed that for high inoculum levels (10^8 CFU/mL) planktonic growth can be used to approximate colony growth.

Growth dynamics of foodborne pathogens and spoilage microorganisms have been studied on the surface and within specific solid(like) food products, e.g., seafood (Mejlholm and Dalgaard, 2007), lettuce (Koseki and Isobe, 2005), pâté (Farber et al., 1995), chicken (Noriega et al., 2010a), meat products (Devlieghere et al., 2001), eggs (Ohkocki et al., 2013), and, in some cases, predictive models have been built for these specific case studies. On the other hand, some research groups have focused more on obtaining fundamental knowledge about food (microstructure) and colony dynamics in food model systems (Antwi et al., 2006; Aspidou et al., 2014; Boons et al., 2014; Mertens et al., 2011; Noriega et al., 2010b; Stecchini et al., 1998, 2004; Theys et al., 2008), the effect of solid structure for both growth kinetics (Lebert et al., 1998; Skandamis et al., 2007; Stecchini et al., 1998) and the probability of growth of certain pathogens, such as *Listeria monocytogenes*

(Koutsoumanis et al., 2004). The difference in growth behavior compared to a liquid system suggests that growth in solid systems may not be well predicted by models derived from liquid systems (Brocklehurst et al., 1997).

In this paper, growth dynamics of *Salmonella* Typhimurium and *Escherichia coli* K12 were estimated as a function of temperature for different growth morphologies. Microbial growth was studied in a liquid system promoting planktonic growth and a solid system prepared by adding gelatin (5% (w/v)) to the corresponding liquid system, yielding either immersed or surface colonies. *S. Typhimurium* and *E. coli*, two gram-negative bacteria, were selected as target microorganisms since both food-borne pathogens are reported to yearly cause a high level of food-borne outbreaks (EFSA and ECDC, 2014). While *E. coli* was grown under optimal conditions in terms of pH and water activity (a_w), for *S. Typhimurium* pH and a_w were adjusted to 5.5 and 0.990 in order to also assess the effect of a slightly stressing environment often found in real foods. Seven temperatures in the suboptimal temperature range [8.0°C- 22.0°C], mimicking normal and temperature-abuse storage conditions, were selected for the growth experiments. The lower boundary of the temperature region considered was selected such that differentiation between growth and lag/no-growth is clear. For the upper boundary, room temperature conditions were selected. The effect of temperature on the growth rate was evaluated for the different growth morphologies by fitting the data with the square root model (Ratkowsky et al., 1982) and comparing values of the two model parameters.

2. MATERIALS AND METHODS

2.1 Microorganisms and preculture conditions

A stationary phase culture of *Salmonella enterica* serovar Typhimurium SL1344, kindly provided by the Institute of Food Research (IFR, Norwich, UK), was stored at -80°C in Tryptone Soya Broth (TSB (Oxoid Ltd., Basingstoke, UK)) supplemented with 25% (v/v) glycerol (Acros Organics, NJ, USA). For every experiment, a purity plate was prepared by spreading a loopful of the frozen stock culture onto

a Tryptone Soya Agar plate (TSA (Oxoid Ltd., Basingstoke, UK)) and incubated at 37.0°C for 24 h. In a first step, one colony from the purity plate was transferred into 20 mL TSB and incubated at 37.0°C for 8 h under static conditions (Binder KB-series incubator; Binder Inc., NY, USA). Afterwards, 200 µL of the stationary phase culture was added to 20 mL of fresh TSB and incubated for 16 h under the same conditions.

Escherichia coli K12 MG1655 was acquired from the *E. coli* Genetic Stock Center at Yale University and stored at -80°C in Brain Heart Infusion (BHI) broth (Oxoid Ltd., Basingstoke, UK) supplemented with 25% (v/v) glycerol. A purity plate from this stock culture was prepared on BHI supplemented with 1.2% (w/v) agar (BHIA; Agar technical n°3, Oxoid Ltd., Basingstoke, UK) and incubated for 24 h at 37.0°C. Similarly to *S. Typhimurium*, one colony was transferred to 20 mL BHI, incubated for 8 h at 37.0°C under static conditions, refreshed in BHI and incubated under the same conditions for 16 h. Cell cultivation under the above defined conditions yielded early-stationary phase populations for both *S. Typhimurium* and *E. coli* with approximately a cell density of 10⁹ CFU/mL. These cell cultures were used to inoculate the corresponding growth media.

2.2 Liquid systems: preparation and growth conditions

In this section the preparation of the liquid systems and the growth conditions for both microorganisms are described.

For the experiments with *S. Typhimurium*, TSB without dextrose (Becton, NJ, USA) was adjusted to a pH value of 5.5 (pH meter; Documeter, Sartorius, Göttingen, Germany) by the addition of 5 M HCl (Acros Organics, NJ, USA), and an *a_w* value of 0.990 (*a_w* meter; AWK-40, Nagy, Gäufelden, Germany) by the addition of NaCl (Sigma Aldrich, MO, USA). The amount of NaCl to be added was determined based on the calibration curve as reported in Theys et al. (2008). For *E. coli*, a BHI solution at pH 7.4 and *a_w* 0.995 was directly used for the growth experiments.

Experiments in liquid systems were performed in petri dishes filled with 20 mL of the corresponding growth medium, cells with an initial cell level of approximately 10³ CFU/mL. This initial cell

concentration was obtained by serial decimal dilution of the corresponding preculture with dilution medium at the same pH and a_w as the further growth medium. Then 0.25 mL of the last dilution was added into 250 mL of the growth medium and after shaking dispensed in petri dishes. For each temperature, 20-30 petri dishes were placed in a temperature controlled incubator (KB 8182, Termaks, Bergen, Norway) at [8.0°C-22.0°C] without shaking. At regular time instants, a petri dish was removed for further microbiological analysis.

2.3 Gelified systems: preparation and growth conditions

This section focuses on the preparation of gelified systems with immersed and surface colonies of *S. Typhimurium* and *E. coli*.

Gelatin at 5% (w/v) concentration (gelatin from bovine skin, type B, Sigma-Aldrich, MO, USA) was added to TSB or BHI, depending on the microorganism, to obtain the solid systems. After heating the media for about 20 min in a water bath (GR150-S12, Grant Instruments Ltd, Shepreth, UK) at 60.0°C, the gelatin melted and the pH and a_w were adapted to 5.5 and 0.990 for *S. Typhimurium*. For *E. coli*, the pH (6.7) and a_w (0.995) were left unchanged, being around the optimal values for *E. coli* growth. The gelled media for both microorganisms were filter-sterilized using a 0.2 μ m filter (Filtertop, 150 mL, 0.22 μ m, TPP, Switzerland). In order to grow either surface or immersed colonies of both microorganisms, two different experimental procedures were implemented. To obtain immersed colonies, the corresponding growth medium supplemented with gelatin was kept liquid by heating at 37.0°C in a water bath and then, inoculated homogeneously by shaking with 10^3 CFU/mL. Hereafter, a set of 20-30 petri dishes was filled with 20 mL of this mixture by using a sterile pipette. These plates were stored in a refrigerator (4.0°C) for about 15 minutes in order to solidify the gelatin mixture. When gels solidified, 10 mL of liquid cell-free gelatin media was added on top of the solid gelatin layer already in the petri dish to prevent outgrowth of surface colonies. In order to solidify this added top layer, a short storage period at 4.0°C followed. Finally, all petri dishes were sealed with parafilm to prevent evaporation and placed simultaneously in a temperature controlled incubator without

shaking at the same temperatures assigned for liquid systems. At regular time moments, plates were removed from the incubator and the corresponding microbiological analyses were performed.

To grow surface colonies, 20 mL of non-inoculated growth media supplemented with gelatin, kept liquid by heating at 60.0°C, was pipetted into sterile petri dishes. After solidification of the gelatin mixture at 4.0°C, the plates were surface inoculated at approximately 10^3 CFU/mL, with 50 µL of the corresponding diluted preculture. After sealing, all plates were placed simultaneously in a temperature controlled incubator at the mentioned temperatures. Plates were piled in order to prevent possible condensation on the surface of all plates, which could turn an apparently immobilized surface colony into a liquid surface film. The top plates, possibly condensed, were not used for further sampling. Plates were removed from the incubator at regular time instances for microbiological analysis.

2.4 Cell recovery and microbiological analysis

The evolution of cell density towards time was determined via viable plate counting for the different tested conditions. For experiments in liquid systems, 1 mL was taken homogeneously from the petri dish by pipetting up and down several times and serial dilutions were made in the corresponding dilution media. For experiments in the gelified model system, the content of the petri dish was transferred to a stomacher bag with a sterile spoon and homogenized in the stomacher after short liquefaction of the medium at 37.0°C. 1 mL was extracted from this bag and serial dilutions in the appropriate dilution media were made. For all samples, 2-4 dilutions were plated onto either TSA or BHIA plates using a spiral plater (Eddy-Jet, IUL Instruments, Barcelona, Spain). Plates were placed at 37.0°C during 24 h before counting. Cell counts shown in the figures are the mean of all countable dilutions and replicates for each sample.

2.5 Modeling and parameter estimation

Experimental data were fitted with the primary growth model of Baranyi and Roberts (1994):

$$\begin{aligned}\frac{dN(t)}{dt} &= \frac{Q(t)}{1+Q(t)} \cdot \mu_{max} \cdot \left(1 - \frac{N(t)}{N_{max}}\right) \cdot N(t) \\ \frac{dQ(t)}{dt} &= \mu_{max} \cdot Q(t) \\ \lambda &= \frac{\ln\left(1 + \frac{1}{Q_0}\right)}{\mu_{max}}\end{aligned}\tag{1}$$

where $N(t)$ [CFU/mL] is the cell density at time t [h], N_{max} [CFU/mL] is the maximum cell density at the stationary phase, μ_{max} [h^{-1}] is the maximum growth rate and $Q(t)$ [-] is a measure of the physiological state of the cells.

To describe the relationship between temperature and maximum growth rate in the entire biokinetic temperature range, Ratkowsky et al. (1982) developed the following square root (SQRT) model:

$$\sqrt{\mu_{max}} = b \cdot (T - T_{min})\tag{2}$$

with b [$^{\circ}\text{C}^{-1} \text{ h}^{-1/2}$], a regression coefficient and T_{min} [$^{\circ}\text{C}$], the (conceptual) minimum temperature for growth.

Parameters of the Baranyi and Roberts model and the SQRT model were estimated via the minimization of the sum of square errors

$$SSE = \sum (y_{exp}(t_i) - y_{mod}(t_i, \mathbf{p}))^2\tag{3}$$

with $y_{exp}(t_i)$ and $y_{mod}(t_i, \mathbf{p})$ the experimental measurement and the model simulation respectively at time t_i for the Baranyi and Roberts model, or temperature T_i for the SQRT model. The vector $\mathbf{p}$ contains the parameters to be estimated. For the Baranyi and Roberts model, this includes $N(0)$, $Q(0)$, μ_{max} , and N_{max} . For the square root model, the parameters to be estimated are T_{min} and b . The minimum SSE was determined using the *lsqnonlin* routine of the Optimization Toolbox of Matlab (The Mathworks Inc.). Standard errors of parameter estimates were calculated from the Jacobian matrix.

2.6 Statistical analysis

Analysis of variance (ANOVA) test was performed to determine whether there are any significant differences amongst means of logarithmically transformed viable counts, at a 95.0% confidence level ($\alpha = 0.05$). Fisher's Least Significant Difference (LSD) test was used to distinguish which means were significantly different from which others. Standardized skewness and standardized kurtosis were used to assess if data sets came from normal distributions. These analyses were performed using Statgraphics Centurion XVI.I Package (Statistical Graphics, Washington, USA). Test statistics were regarded as significant when P was ≤ 0.05 .

3. RESULTS

Figures 1 and 2 present the growth curves of *S. Typhimurium* and *E. coli* at 8.0, 10.0, 12.5, 15.0, 17.5, 20.0 and 22.0°C fitted with the growth model of Baranyi and Roberts (1994). Different growth morphologies, i.e., planktonic cells, immersed colonies and surface colonies are considered. Resulting lag times, maximum growth rates and maximum cell densities, and the corresponding statistical analyses are listed in Table 1. For *S. Typhimurium* at 8.0°C the experiments were not very reproducible due to the stressing conditions, which results in high standard errors for most of the growth parameters.

Lag phase. When studying the duration of the lag phase for both microorganisms at the different temperatures, almost no significant differences between the three growth morphologies are observed. The only statistically significant difference is found for *S. Typhimurium* at 22.0°C, where immersed colonies have a shorter lag phase. Even though there are no statistical differences between the growth morphologies at most temperatures and for both microorganisms, lag time values at lower temperatures are quite varied when liquid and gelified systems are compared. The lack in statistical difference is due to very large standard errors. For both *S. Typhimurium* and *E. coli*,

the duration of the lag phase slightly decreases as the temperature increases, although, from 12.5°C and above, no further significant decrease of the lag phase is observed.

Maximum growth rate. For *S. Typhimurium*, the different growth morphologies have similar maximum specific growth rates at most temperatures. However, at 10.0°C planktonic cells grow faster than colonies. At higher temperatures, (surface) colonies tend to have the highest μ_{max} . For *E. coli*, significant differences between the three morphologies are mainly situated at the lower temperatures. More specifically at 8.0 and 10.0°C, (small) differences in the slope of the exponential phase can be observed, with planktonic cells growing faster than both types of colonies. At higher temperatures, growth curves have similar slopes, also indicated by statistically identical parameter values for μ_{max} . In Figure 3, the square root of the maximum specific growth rate is plotted as a function of temperature. Due to this mathematical transformation, standard errors might seem high although for the real μ_{max} , standard errors are not significant. For more representative values, Table 1 can be consulted. When the maximum growth rate is plotted as a function of the temperature for each microorganism, differences in μ_{max} between the growth morphologies appear rather limited (see Figure 3). When comparing maximum growth rates at the different temperatures for one microorganism, no clear trend can be observed between the different growth morphologies, i.e., the highest μ_{max} value at the different temperatures is not always presented for the same growth morphology. As extensively reported in literature, if temperatures increase growth rates for all morphologies will increase, as proved for the two microorganisms studied.

The maximum growth rates were fitted with the square root model of Ratkowsky et al. (1982) as a function of temperature for the different microorganisms and growth morphologies (see Figure 3). Corresponding b and T_{min} values are presented in Table 2. For each microorganism, data for the three different growth morphologies are fitted separately. For *S. Typhimurium*, no significant differences between the growth morphologies can be observed for the parameter b . For *E. coli*, immersed colonies have a higher b value from a statistically point of view, although the mean value is relatively similar to the value for planktonic cells and surface colonies. The minimum temperatures for the

different growth morphologies are identical in the case of *S. Typhimurium*. Similar to the coefficient b , slightly higher values for T_{min} are found for the immersed colonies for the experiments with *E. coli*. But again, the mean values for this parameter are in the same range. In a second step experimental data from all three growth morphologies are combined and fitted, resulting in identical values as for the individual growth morphologies for both b and T_{min} .

Maximum cell density. Regarding the maximum cell density, no very clear trend is observed for either of the studied microorganisms. For *S. Typhimurium*, the biggest differences can be found at the higher temperatures, where the order seems to be $N_{max(surface)} \geq N_{max(planktonic)} \geq N_{max(immersed)}$. For *E. coli*, significant differences can be found at the boundaries of the tested range of temperatures. Surface colonies seem to have the highest maximum cell densities, while immersed colonies again reach a lower N_{max} . For both microorganisms, even though no general trend is deducted concerning the different growth morphologies, if temperature increases a slight increase in N_{max} is noticeable.

4. DISCUSSION

In the present work, the effect of cell immobilization on the growth dynamics of *S. Typhimurium* and *E. coli* is studied as a function of temperature (8.0°C to 22.0°C).

Three different scenarios are considered: (1) a planktonic culture growing in a liquid environment, (2) immersed colonies, and (3) surface colonies. Growth parameters were estimated with the model of Baranyi and Roberts (1994). Subsequently, maximum growth rates were fitted as a function of temperature with the square root model of Ratkowsky et al. (1982).

Lag phase. Regarding the lag phase estimates, it is overall observed that there is no statistically significant difference in lag duration between colonies and planktonic cells. This observation is in contrast with the results published by Knudsen et al. (2012), where for *S. Typhimurium* grown at 25°C in gel cassettes, the lag phase for colonies is significantly longer than the one for planktonic cells. Differences between Knudsen et al. (2012) and the present work can probably be accounted to the

use of different experimental set-ups. As stated in this study, Boons et al. (2013) observed similar lag phase durations in both liquid systems and different gelled systems for *S. Typhimurium* and *E. coli* at 23.5°C at low salt concentrations. In the present study, the only significant difference considering the lag phase was observed for *S. Typhimurium* at 22°C, where the following order was observed: $lag_{(immersed\ colonies)} < lag_{(surface\ colonies)} \leq lag_{(planktonic\ cells)}$. This is again in line with Boons et al. (2013), where for higher salt concentrations (comparable to the amount added to the TSB(/gelatin) system used in this work for *S. Typhimurium*), lag phases were found longer for the liquid system, as compared to all gelled systems. This result was explained by interactions between the gelatin and the sodium chloride, causing the actual concentration of ions detected by the cells to be lower as compared to liquid systems. For the gelled systems, this buffering capacity of the gelatin results in a smaller osmotic imbalance in the cells, caused by the transfer from the preculture to the growth medium. In the liquid system, larger osmotic imbalances caused by a larger change in ionic concentration, cause delays in cell division, explaining a longer lag phase duration (Boons et al., 2013; Jovanovich et al., 1988; Roth et al., 1985).

Maximum growth rate. For both microorganisms, statistically significant differences in μ_{max} were discovered between the different growth morphologies at certain temperatures closer to the limits of the studied temperature range. At the lower (most stressing) temperatures planktonic cells of both microorganisms exhibit the highest growth rate. However, the trend at the higher temperatures involves higher values of μ_{max} for surface colonies. In general, these differences between the growth morphologies are limited for most temperatures and planktonic cells and colonies present similar maximum growth rates. This is in contrast with most literature addressing the growth dynamics of planktonic cultures and surface and/or immersed colonies, where the main observation with respect to the maximum growth rate is the following: $\mu_{planktonic} \geq \mu_{immersed} \geq \mu_{surface}$ (see, e.g., Aspidou et al., 2014; Brocklehurst et al., 1997; Meldrum et al., 2003; Theys et al., 2008). In the work of Brocklehurst et al. (1997) and Meldrum et al. (2003), this effect was observed for *S. Typhimurium* and *Listeria monocytogenes*, for different concentrations of salt, sucrose (or a_w) or several pH values. Theys et al.

(2008) observed that the addition of 1% and 5% (w/v) gelatin resulted in a significant reduction of the growth rate of *S. Typhimurium* at 20.0°C for a certain pH and a_w range, including those values used for *S. Typhimurium* in this study. Aspidou et al. (2014) studied the growth dynamics of *Listeria monocytogenes* in liquid substrates and gels of sodium alginate and gelatin at different temperatures. Growth in gels proved to be slower than for the liquid systems with a most pronounced effect at low temperatures, indicating a more significant effect of the microstructure at these low temperatures. This trend is also visible in the present study, where the most pronounced differences between the growth morphologies are found at the lower temperatures and even the expected order $\mu_{\text{planktonic cells}} \geq \mu_{\text{colonies}}$ is respected. This indicates a synergy between the cold shock and the solid environment, resulting in lower growth rates for the colonies, on the basis of the hurdle technology principle. More specifically, when stress factors are combined (e.g., low temperatures and a solid environment), a synergistic inhibitory effect on microbial growth is often observed and growth is inhibited under less stressful conditions than when each of the stress factors would be applied on its own (Leistner, 2000). A decreasing temperature has an effect on both the microorganisms and the solid support. From one side, the cell membrane of the bacteria alters due to the cold shock, resulting in a higher flexibility, which is known as the homeoviscous adaptation (Sinensky, 1974; Van Derlinden and Van Impe, 2012). From the other side, the microorganism responds to the cold shock by a metabolic switch to the production of specific shock proteins (Van Derlinden and Van Impe, 2012). Moreover, at low temperatures the rigidity of the polymer chain of the gelatin increases and the molecular mobility decreases, leading to more mechanical restraints from the gelatin network on the microorganisms (Ferry, 1948). Still, in this work major differences between the three growth morphologies remain limited. It has to be pointed out that in the above works, planktonic cultures were grown under shaken conditions. Shaken vs. static microbial cultures probably account for most of the differences between a liquid and solid environment. The divergence in growth dynamics between shaken or static samples can, most likely, be explained by a difference in transport of nutrients, metabolites and oxygen from and to the cells. In static systems,

also cell sedimentation takes place with culture time, influencing the distribution of nutrients and metabolites. Among these phenomena, the difference in transport of oxygen is possibly the most influencing one. Theys et al. (2008) reported that within a shaken and thus perfectly mixed TSB broth in an Erlenmeyer, a certain oxygen concentration is maintained throughout the whole culture. However, when liquid cultures are not shaken (e.g., in a petri dish), the oxygen concentration drops beyond the detection limit. Since an important difference between maximum growth rates was observed for experiments in liquid systems performed in Erlenmeyers and petri dishes, Theys et al. (2008) concluded that the significant drop in μ_{max} resulted from not shaking the liquid system. As compared to the static liquid system, Theys et al. (2008) measured identical low oxygen concentrations within a gelatin gel (5% (w/v)) in a petri dish. According to Theys et al. (2008), oxygen availability proved to be more important than the medium solidness and the resulting immobilization of colonies due to the solid medium. In the present study, low oxygen concentrations in both the non-shaken liquid systems and the gelled systems could explain the similar maximum growth rates of planktonic cells and colonies. This assumption can be confirmed by the work of Knudsen et al. (2012), who also observed identical maximum growth rates for planktonic cells and colonies, cultured in (non-shaken) IFR gel cassettes. The above results prove that under certain growth conditions, the effect of the microstructure is negligible or less pronounced. Static conditions limit oxygen transport, causing reduced growth rates in the liquid system as compared to most literature and lead to similar μ_{max} values to the colonies. Only when additional stress is created (e.g., low temperatures), the effect of the microstructure is more pronounced.

In a next step, the growth rates were fitted as a function of temperature with the square root model. Very similar values for the b and T_{min} parameters were obtained for the three growth morphologies, which cannot be statistically differentiated (see Table 2 and Figure 3). The minimum growth temperature for *S. Typhimurium*, at pH 5.5 and a_w 0.990, is estimated at approximately 6.0-7.0°C. For *E. coli*, at more optimal conditions, T_{min} is estimated between 5.0 and 6.0°C. Both estimations can be

considered realistic and corresponds with values reported in literature (*S. Typhimurium* (ICMSF (1996)); *E. coli* (ICMSF (1996); Nauta and Dufrenne (1999); Rosso et al. (1993)).

Maximum cell density. Regarding the maximum cell density N_{max} , in general the order $N_{max(surface)} > N_{max(planktonic)} > N_{max(immersed)}$ was observed for both microorganisms. This is in line with the work of Robins and Wilson (1994), who concluded that the maximum cell density for immersed colonies is lower than N_{max} for liquid cultures of *Yersinia enterocolitica*. Since in this work none of the systems were shaken, higher oxygen concentrations in the case of surface colonies might explain why in most cases their maximum cell concentration is the highest.

In summary, this work shows that, for the selected environmental conditions, the growth morphology (planktonic vs. colony growth) has a negligible effect on the growth dynamics of *S. Typhimurium* and *E. coli* as a function of temperature. This observation differs from literature where, in most cases, a clear difference in growth rate is observed between planktonic cells and colonies of *S. Typhimurium* and *E. coli*. However, it is very likely that this difference is due to different experimental set-ups. In most literature, liquid cultures are shaken, which results in a well-aerated and nutrient-rich environment, in contrast to gel systems that are subjected to nutrient and oxygen diffusion limitations. In this work, both liquid and gel systems are incubated under static conditions, which could explain the similar results obtained for planktonic, surface and immersed colonies. This work relies on the viable plate count technique to monitor microbial concentrations, so that the overall population growth dynamics are estimated rather than the colony dynamics. In order to further study the behavior of a single colony, non-invasive in-situ techniques, such as optical density (Mertens et al., 2012) would be required. This technique enables to monitor the colony surface as a function of time. By combining these measurements with viable counts, colony dynamics could be estimated and compared to planktonic growth.

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FIGURES

Figure 1: Growth curves of *Salmonella* Typhimurium at different temperatures: (◆ & (-)) planktonic cells, (x & (-•)) immersed colonies, and (● & (- -)) surface colonies. Symbols correspond to experimental data and lines to the fit of the Baranyi and Roberts model.

experimental data and lines to the fit of the Baranyi and Roberts model.

Figure 2: Growth curves of *Escherichia coli* at different temperatures: (◆ & (-)) planktonic cells, (x & (-•)) immersed colonies, and (● & (- -)) surface colonies. Symbols correspond to experimental data and lines to the fit of the Baranyi and Roberts model.

Figure 3: Maximum specific growth rate of *Salmonella* Typhimurium (left) and *Escherichia coli* (right) as a function of temperature fitted with the square root model (Ratkowsky et al. 1982). (a) (◆ & (-)) planktonic cells, (x & (•-)) immersed colonies, and (● & (- -)) surface colonies. (b) The square root model fitted to all data simultaneously (-): (◆) planktonic cells, (x) immersed colonies, and (•) surface colonies.

TABLES

Table 1: Growth parameters of the Baranyi and Roberts model at different temperatures obtained for planktonic cells, immersed colonies and surface colonies of *S. Typhimurium* and *E. coli*.

strain	T (°C)	Kinetic parameters											
		Lag (h) ¹ ₂			μ _{max} (1/h) ¹ ₂			N _{max} (CFU/mL) ¹ ₂			RMSE ³		
		Planktonic cells	Immersed colonies	Surface colonies	Planktonic cells	Immersed colonies	Surface colonies	Planktonic cells	Immersed colonies	Surface colonies	Planktonic cells	Immersed colonies	Surface colonies
Salmonella Typhimurium	8.0	0.0 ± 178.6 ^a _A	100.0 ± 150.5 ^a _A	59.4 ± 262.5 ^a _A	0.008 ± 0.002 ^a _A	0.007 ± 0.001 ^a _A	0.007 ± 0.002 ^a _A	15.0 ± 0.7 ^a _A	15.0 ± 0.8 ^a _A	17.4 ± 1.7 ^b _A	1.320	1.147	2.090
	10.0	66.2 ± 16.7 ^a _A	0.0 ± 80.2 ^a _A	0.0 ± 58.5 ^a _A	0.036 ± 0.002 ^b _B	0.018 ± 0.002 ^a _A	0.021 ± 0.002 ^a _A	19.6 ± 0.2 ^a _B	21.2 ± 0.9 ^a _C	20.1 ± 0.5 ^a _B	0.677	1.484	1.244
	12.5	0.0 ± 11.3 ^a _A	0.0 ± 9.6 ^a _A	0.0 ± 10.3 ^a _A	0.068 ± 0.005 ^a _C	0.080 ± 0.007 ^a _B	0.072 ± 0.005 ^a _B	20.9 ± 0.4 ^a _C	20.6 ± 0.3 ^a _{BC}	21.3 ± 0.3 ^a _{BC}	0.634	0.567	0.587
	15.0	3.3 ± 1.9 ^a _A	0.0 ± 2.1 ^a _A	0.0 ± 2.2 ^a _A	0.245 ± 0.010 ^a _D	0.266 ± 0.018 ^a _C	0.253 ± 0.011 ^a _C	20.5 ± 0.2 ^{ab} _C	19.9 ± 0.2 ^a _B	21.0 ± 0.2 ^b _{BC}	0.356	0.384	0.413
	17.5	3.8 ± 1.6 ^a _A	2.3 ± 1.0 ^a _A	2.2 ± 1.1 ^a _A	0.329 ± 0.015 ^a _E	0.395 ± 0.010 ^b _D	0.378 ± 0.011 ^b _D	20.8 ± 0.1 ^b _C	20.3 ± 0.1 ^a _B	21.2 ± 0.1 ^c _{BC}	0.321	0.245	0.247
	20.0	3.2 ± 1.0 ^a _A	2.2 ± 0.8 ^a _A	4.5 ± 1.1 ^a _A	0.486 ± 0.016 ^a _F	0.482 ± 0.013 ^a _E	0.518 ± 0.023 ^a _E	20.6 ± 0.1 ^b _C	20.1 ± 0.1 ^a _B	21.3 ± 0.2 ^c _{BC}	0.318	0.352	0.290
	22.0	3.6 ± 0.6 ^b _A	0.7 ± 0.7 ^a _A	2.9 ± 0.5 ^b _A	0.626 ± 0.016 ^a _G	0.535 ± 0.015 ^a _F	0.635 ± 0.015 ^b _F	20.8 ± 0.1 ^b _C	20.0 ± 0.1 ^a _B	21.6 ± 0.1 ^c _C	0.257	0.296	0.243
Escherichia coli	8.0	41.5 ± 36.9 ^a _{BC}	0.0 ± 87.9 ^a _A	54.6 ± 38.4 ^a _B	0.016 ± 0.001 ^a _A	0.012 ± 0.001 ^a _A	0.015 ± 0.001 ^{ab} _A	20.3 ± 0.2 ^a _A	17.1 ± 0.4 ^b _A	19.8 ± 0.2 ^b _A	0.676	1.082	0.859
	10.0	69.1 ± 9.3 ^a _C	0.0 ± 41.5 ^a _A	0.0 ± 19.0 ^a _A	0.047 ± 0.002 ^b _B	0.027 ± 0.002 ^a _A	0.033 ± 0.002 ^a _A	20.6 ± 0.1 ^a _{ABC}	20.1 ± 0.3 ^a _{BC}	21.0 ± 0.2 ^b _{BC}	0.506	0.968	0.619
	12.5	12.8 ± 7.1 ^a _{AB}	0.0 ± 12.0 ^a _A	0.0 ± 7.0 ^a _A	0.079 ± 0.004 ^a _C	0.069 ± 0.005 ^a _B	0.078 ± 0.004 ^a _B	20.6 ± 0.2 ^a _{ABC}	20.7 ± 0.3 ^a _{CD}	21.5 ± 0.2 ^b _C	0.641	0.911	0.591
	15.0	4.7 ± 3.0 ^a _A	1.3 ± 1.5 ^a _A	0.0 ± 4.2 ^a _A	0.178 ± 0.009 ^a _D	0.179 ± 0.005 ^a _C	0.179 ± 0.011 ^a _C	20.4 ± 0.4 ^a _{AB}	20.1 ± 0.2 ^a _{BC}	20.7 ± 0.5 ^a _B	0.497	0.235	0.638
	17.5	4.3 ± 1.0 ^a _A	0.0 ± 2.5 ^a _A	0.0 ± 1.8 ^a _A	0.290 ± 0.006 ^b _E	0.272 ± 0.013 ^{ab} _D	0.245 ± 0.008 ^a _D	21.2 ± 0.1 ^a _D	20.7 ± 0.3 ^a _{CD}	20.8 ± 0.2 ^a _B	0.215	0.496	0.335
	20.0	3.0 ± 1.3 ^a _A	0.0 ± 2.2 ^a _A	1.3 ± 1.5 ^a _A	0.389 ± 0.016 ^a _F	0.358 ± 0.018 ^a _E	0.361 ± 0.015 ^a _E	21.0 ± 0.2 ^a _{CD}	20.7 ± 0.2 ^a _D	21.0 ± 0.2 ^a _{BC}	0.561	0.689	0.684
	22.0	0.0 ± 0.6 ^a _A	0.0 ± 1.0 ^a _A	0.0 ± 1.4 ^a _A	0.495 ± 0.009 ^a _G	0.502 ± 0.017 ^a _F	0.460 ± 0.017 ^a _F	20.8 ± 0.1 ^b _{BCD}	20.0 ± 0.1 ^a _B	21.0 ± 0.2 ^b _{BC}	0.220	0.367	0.467

¹ For each microorganism and temperature level, parameters of the Baranyi and Roberts model bearing different superscripts (no lowercase letters in common) are significantly different ($P \leq 0.05$)

² For each microorganism and growth morphology, parameters of the Baranyi and Roberts model bearing different subscripts (no uppercase capital letters in common) are significantly different ($P \leq 0.05$)

³ Root Mean Squared Error

Table 2: Parameters of the square root model for planktonic cells, immersed colonies, surface colonies of *S. Typhimurium* and *E. coli*.

Strain	Cell Morphology	Parameters square root model		
		$b (^{\circ}\text{C}^{-1}\text{h}^{-1/2})^1$	$T_{\min} (^{\circ}\text{C})^1$	RMSE ²
<i>Salmonella</i> <i>Typhimurium</i>	Planktonic cells	0.0513 ± 0.0027^a	6.38 ± 0.52^a	0.0341
	Immersed colonies	0.0510 ± 0.0046^a	6.39 ± 0.89^a	0.0586
	Surface colonies	0.0543 ± 0.0032^a	6.75 ± 0.56^a	0.0404
	Global	0.0522 ± 0.0019^a	6.51 ± 0.35^a	0.0413
<i>Escherichia</i> <i>coli</i>	Planktonic cells	0.0418 ± 0.0013^a	5.06 ± 0.35^a	0.0167
	Immersed colonies	0.0436 ± 0.0018^b	5.86 ± 0.41^b	0.0222
	Surface colonies	0.0408 ± 0.0012^a	5.27 ± 0.32^a	0.0153
	Global	0.0421 ± 0.0009^a	5.41 ± 0.23^a	0.0196

¹ For each microorganism, parameters of the SQRT model bearing different superscripts (no letters in common) are significantly different ($P \leq 0.05$)

² Root Mean Squared Error