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Influence of the growth morphology on the behavior of

Salmonella* Typhimurium and *Listeria monocytogenes

under osmotic stress

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ABSTRACT

The microbial growth morphology, as a consequence of the food structure, has been acknowledged to play a key role on the growth behavior. While in liquid media planktonic growth is observed, in a solid(like) environment cells are immobilized and forced to grow as (surface) colonies. This immobilization could affect the cell physiology and metabolism. Apart from the growth morphology, other intrinsic factors influence microbial growth. Osmotic stress and acidic stress have an impact on microbial growth. Moreover, the combination of intrinsic factors could result in a synergetic inhibitory effect on the growth behavior (hurdle technology). In this paper, the growth dynamics of *Salmonella* Typhimurium and *Listeria monocytogenes* under stressing conditions are studied for two different growth morphologies, i.e., (i) planktonic cells and (ii) surface colonies. Microorganisms are grown in petri dishes, incubated at 20°C under static conditions. To create the solid(like) environment, 5% (w/v) gelatin is added. Stressing growth conditions are created by adding salt [0-8% (w/v)] and adapting the pH [5.5-7.0]. Cell density is determined via the viable plate count technique. While the studied pH-range has a negligible effect, the addition of salt significantly reduces growth. The growth morphology, resulting from the intrinsic food (model) structure, affects the microbial growth dynamics under static incubation conditions. In contrast to literature, surface colonies have higher or similar maximum specific growth rates than planktonic cells under most of the selected experimental conditions. For example, for *S. Typhimurium* at pH 6.5 and 2% (w/v) NaCl, planktonic cells have a maximum specific growth rate of 0.435 1/h while μ_{max} for surface colonies has a value of 0.464 1/h. For *L. monocytogenes* at pH 6.0 and 0% (w/v) NaCl, $\mu_{max, planktonic cells}$ is 0.375 1/h and $\mu_{max, surface colonies}$ is 0.424 1/h. This is due to limited oxygen availability as a result of the experimental protocol implemented, leading to lower μ_{max} values for planktonic cells as opposed to μ_{max} values for shaken cultures in other studies appearing in literature. This indicates that under static incubation, the effect of the microstructure is often negligible as compared to the oxygen availability. However,

for the most stressing experimental conditions, the combination of high salt concentrations and a solid(like) growth environment inhibits growth, and the order $\mu_{max, planktonic\ cells} \geq \mu_{max, surface\ colonies}$ is respected. This is for instance illustrated in case of *S. Typhimurium* at pH 5.5 and 6% (w/v) NaCl: $\mu_{max, planktonic\ cells}$ reaches a value of 0.183 1/h while $\mu_{max, surface\ colonies}$ is only 0.142 1/h. Also for *L. monocytogenes* at pH 6.0 and 8% (w/v) NaCl, $\mu_{max, planktonic\ cells}$ (0.145 1/h) is higher than $\mu_{max, surface\ colonies}$ (0.113 1/h). This study indicates the relevance of intrinsic factors on the growth dynamics and addresses the importance to include growth morphology, determined by the intrinsic food structure, in predictive models.

Keywords: food structure; immobilization; surface colony; planktonic cells; osmotic stress; acidic stress

1. INTRODUCTION

Microbial behavior in food is influenced by different factors which can be divided into two main groups. On the one hand, extrinsic factors or environmental conditions, like temperature or storage atmosphere. On the other hand, intrinsic factors or physicochemical properties of the food or growth medium itself play an important role. These include available nutrients, medium pH and water activity (e.g., influenced by the presence of salt in the medium) or food structure. Next to these two main groups, also interactions between microorganisms (implicit factors) and some processing factors (like washing or packaging of food) can influence the growth dynamics (Adams and Moss, 2006). The hurdle technology is a strategy to ensure food safety by combining the above (preservative) factors. When stressing factors are combined, a synergistic inhibitory effect on microbial growth is often observed and growth can be inhibited under less stressful conditions than for each stress factor applied separately (Baka et al., 2013; Bidlas and Lambert, 2008; Leistner, 2000).

For many decades, predictive microbiology focused on studying microbial behavior in liquid systems. However, a solid structure has been acknowledged to play a key role on microbial growth. Being an important intrinsic factor, food structure influences the growth morphology of microbial cells. When grown in a liquid environment, cells are freely-dispersed. A local uniform environment is created due to a convective transport of nutrients, metabolites and oxygen (Dens and Van Impe, 2001; McMeekin et al., 2002). In a solid(like) structure, microorganisms are immobilized by the environment forcing them to grow as colonies (Brocklehurst et al., 1997; Wilson et al., 2002). The main transport system in a solid(like) environment is based on diffusion. Slower diffusion transport limits nutrient and oxygen delivery and metabolite removal, resulting in the formation of concentration gradients around colonies (Antwi et al., 2006; Malakar et al., 2000; Wimpenny and Coombs, 1983). The additional stress on the colonies, created by the solid(like) environment, results in slower growth together with a confinement of the growth domain (Antwi et al., 2006; Aspridou et al., 2014; Noriega et al., 2010; Theys et al., 2008). Next to the direct effect of immobilization induced by the solid structure, the location where colonies grow plays an important role on the growth behavior. Two different types of colonies exist: surface colonies growing on the surface of a food product or immersed colonies, which form inside the solid food matrix (Wimpenny, 1992). Literature states that diffusion limitations are higher for growth on a solid surface, resulting in decreased growth rates for surface colonies as compared to immersed colonies or planktonic cells. When growth rates for the different systems are compared, the following order is deducted: broth $\geq$ immersed colonies $\geq$ surface colonies (Brocklehurst et al., 1997). This results in the fact that growth in solid(like) systems may not be well predicted by models derived from broth systems and new models need to be developed to improve microbial safety in the food industry (Kapetanakou et al., 2011; Noriega et al., 2008; Wilson et al., 2002).

Osmotic stress caused by the presence of salt is another intrinsic factor often studied for food preservation. The presence of solutes in a food (model) system, like salt, influences the water activity and therefore, on microbial growth. Growth of bacterial cells requires a controlled flux of water. Cell

exposure to hyperosmotic stress leads to an instantaneous efflux of water from the cell interior, since the bacterial cytoplasmic membrane is permeable to water but not to other metabolites. This results in a reduced turgor pressure and shrinkage of the cytoplasmic volume, together with an increase of the concentration of intracellular metabolites and a reduction of the intracellular water activity. If no active osmotic cell adjustment is feasible, the shrinkage of the cytoplasmic volume would continue until the intracellular water activity equals the extracellular, leading to the impairment of some essential cellular functions (Yousef and Courtney, 2003). However, bacterial cells are able to cope with fluctuations in osmolarity thanks to the development of osmoadaptive strategies, and as a result of the stress exposure may respond in different ways (Noriega et al., 2014; O'Byrne and Booth, 2002; Sleator and Hill, 2001). As an example of an osmoadaptive strategy, literature often reports for *S. Typhimurium* and *L. monocytogenes* that, at salt concentrations > 5% (w/v) NaCl, cell filamentation or elongation takes place (Geng et al., 2003; Hazeleger et al., 2006; Mattick et al., 2000).

The acidity of a food product plays an important role towards the microbial ecology, the rate and the character of its spoilage (Adams and Moss, 2006).

In this work, the growth dynamics of planktonic and colony cells of the gram-negative *S. Typhimurium* and gram-positive *L. monocytogenes* were studied under osmotic and acidic stress at 20°C. These microorganisms have been selected since *S. Typhimurium* is reported to yearly cause a high level of food-borne outbreaks and the fatality rate of *L. monocytogenes* is very high despite its low incidence (EFSA and ECDC, 2014). Two different growth morphologies found in either liquid foods or on product surfaces were assessed: planktonic cells and surface colonies. The solid(like) environment was created by adding 5% (w/v) gelatin. Based on knowledge from previous studies (Smet et al., 2015; Theys et al., 2008), 5% (w/v) gelatin was selected, an amount often found in food (e.g., meat products). The effect of salt and pH on the growth behavior was also assessed. The pH was fixed in the range [5.0-7.0] mimicking pH values found for most food products. For the salt level

a broad range [0-8% (w/v)] was examined, taking into account food products containing very high concentrations of salt (e.g., Blue-veined cheese: 7.5-8.3% (w/w) (Roostita & Fleet, 1996)).

2. MATERIALS AND METHODS

2.1 Experimental plan

A central composite design was used to determine the combined effect of the growth morphology (0 or 5% (w/v) gelatin), pH (5.0-5.5-6.0-6.5-7.0) and sodium chloride concentration (0-2-4-6-8% (w/v)) on the growth dynamics of *S. Typhimurium* and *L. monocytogenes*. The CCD design was chosen as a methodology to have a good span of experiments performed in regions that could capture the microbial responses. It was also chosen in order to reduce the experimental effort if compared with classical full factorial designs. Two central points were used in the CCD, which were pH 6.0, 4% (w/v) NaCl for both planktonic cells and surface colonies. Based on knowledge from previous studies (Smet et al., 2015; Theys et al., 2008), 5% (w/v) gelatin was selected, an amount often found in food (e.g., meat products). The pH was fixed in the range [5.0-7.0] mimicking pH values found for most food products. For the salt level a broad range [0-8% (w/v)] was examined, taking into account very salty food products (e.g., Blue-veined cheese: 7.5-8.3% (w/w) (Roostita & Fleet, 1996)). Optimal growth conditions were also assessed (pH 7.0, 0% (w/v) NaCl) for both growth morphologies and microorganisms.

2.2 Microorganisms and preculture conditions

Salmonella enterica serovar Typhimurium SL1344 was kindly provided by the Institute of Food Research (IFR, Norwich, UK). The culture 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 fresh purity plate was prepared from the frozen stock culture by spreading a loopful

onto a Tryptone Soya Agar plate (TSA (Oxoid Ltd., Basingstoke, UK)) incubated at 37°C for 24 h. One colony from this plate was transferred into 20 mL TSB and incubated under static conditions at 37°C for 8 h (Binder KB series incubator; Binder Inc., NY, USA). Next, 200 µL from this stationary phase culture was added to 20 mL of fresh TSB and incubated under the same conditions for 16 h.

Listeria monocytogenes LMG 13305 was obtained from the Belgian Co-ordinated Collections of Microorganisms (BCCM, Ghent, Belgium). The culture was stored at -80°C in TSB supplemented with 0.6% (w/v) yeast extract (Merck, Darmstadt, Germany) (TSBYE). For each experiment, a new purity plate was prepared on Brain Heart Infusion (BHI (Oxoid Ltd., Basingstoke, UK)) supplemented with 1.2% (w/v) agar (Agar technical n°3, Oxoid Ltd., Basingstoke, UK) and incubated for 24 h at 37°C. One colony from the purity plate was transferred into 20 mL BHI, incubated at 37°C for 8 h under static conditions, refreshed in BHI and incubated again for 16 h.

Cell cultivation under the above defined conditions yielded early-stationary phase populations for both *S. Typhimurium* and *L. monocytogenes*, at about 10⁹ CFU/mL. These were used to prepare the inoculum.

2.3 Liquid systems: preparation and growth conditions

For *S. Typhimurium*, the appropriate amount of salt (0-8% (w/v) NaCl, Sigma Aldrich, MO, USA) was added to TSB without dextrose (Becton, NJ, USA). BHI was used for *L. monocytogenes*. Next to adding the appropriate NaCl amount, the pH was adjusted with a pH meter (DocuMeter, Sartorius, Goettingen, Germany) by the addition of 5M HCl (Acros Organics, NJ, USA). Water activity values (see Table 1) were determined with the aid of an a_w -Kryometer (AWK-40, Nagy, Gaeufelden, Germany).

Experiments in liquid were performed in petri dishes (diameter 5.5 cm) filled with 7 mL of the growth medium and inoculated approximately at 10³ CFU/mL. This cell density was obtained by serial decimal dilutions in a dilution medium with the same pH and salt concentration as the final growth conditions. After shaking, the final inoculated growth medium was dispensed into the petri dishes. For each experiment, 20 petri dishes were placed in a temperature controlled incubator (KB 8182,

Termaks, Bergen, Norway) at 20°C under static conditions. At regular time intervals, petri dishes were removed for further microbiological analysis.

In order to study the effect of this static incubation on experiments in liquid systems, an additional experiment at pH 6.5 and 2% (w/v) NaCl was performed by shaking the petri dishes during incubation at 80 rpm. Preparation of these samples was similar as described above.

2.4 Gelified systems: preparation and growth conditions

Together with the appropriate amount of NaCl, gelatin at 5% (w/v) (gelatin from bovine skin, type B, Sigma-Aldrich, MO, USA) was added to TSB or BHI. After heating for 20 min at 60°C in a thermostatic water bath (GR150-S12, Grant Instruments Ltd, Shepreth, UK) the gelatin melted, the medium was adapted to the appropriate pH and the water activity was measured (Table 1). The medium was then filter-sterilized using a 0.2 µm filter (Filtertop, 150 mL, 0.22 µm, TPP, Switzerland).

In a first step, 7 mL of non-inoculated growth medium supplemented with gelatin and kept liquid at 60°C, was pipetted into sterile petri dishes. After solidification, the plates were surface inoculated at approximately 10^3 CFU/mL (the equivalent of approximately 3.0×10^2 CFU/cm²). This concentration was obtained by serial decimal dilution of stationary phase cells from the second preculture with the appropriate dilution medium, followed by spreading 20 µL of the appropriate dilution onto each petri dish. After being sealed, plates were placed simultaneously in a temperature controlled incubator at 20°C under static conditions. At regular instances, plates were removed from the incubator for sampling.

2.5 Cell recovery and microbiological analysis

The evolution of cell density towards time was determined via viable plate counting. For liquid systems, 1 mL was taken homogeneously from the petri dish by pipetting up and down. Serial decimal dilutions were prepared in the corresponding dilution media (same pH and salt

concentrations as the growth medium). For gelified systems, the content of the petri dish was transferred to a stomacher bag, liquefied in a thermostatic water bath at 37°C and homogenized in the stomacher for 30 seconds. 1 mL was taken from this bag, and serial decimal dilutions were prepared. For all samples, liquid or gelified, 2-4 dilutions were plated (49.2 µL) onto TSA or BHI-Agar plates using a spiral plater (Eddy-Jet, IUL Instruments). Plates were placed at 37°C for 24 h before counting. For all the samples, 3 dilutions were plated. Cell counts shown in the figures are the mean of all countable dilutions for each sample.

2.6 Modelling and parameter estimation

Data were fitted with the Baranyi and Roberts model (1994):

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

where $N(t)$ [CFU/mL] is the cell density at time t [h], N_{max} [CFU/mL] the cell density of the stationary phase, μ_{max} [h⁻¹] the maximum specific growth rate and $Q(t)$ [-] a measure for the physiological state of the cells. For static conditions, the model can be written as:

$$\begin{aligned}\ln(N(t)) &= \ln(N_0) + \mu_{max} \cdot A(t) - \ln\left(1 + \frac{e^{\mu_{max} \cdot A(t)} - 1}{e^{(N_{max} - N_0)}}\right) \\ A(t) &= t + \frac{1}{\mu_{max}} \ln\left(\frac{e^{-\mu_{max} \cdot t} + Q_0}{1 + Q_0}\right)\end{aligned}\quad (2)$$

Parameters of the Baranyi and Roberts model were estimated via the minimization of the sum of square errors (SSE)

$$SSE = \sum_{i=1}^n (y_{exp}(t_i) - y_{mod}(t_i, \mathbf{p}))^2 \quad (3)$$

with $y_{exp}(t_i)$ and $y_{mod}(t_i, \mathbf{p})$ the experimental measurement and the model simulation at time t_i . The vector $\mathbf{p}$ contains the parameters to be estimated ($N(0)$, $Q(0)$, μ_{max} , and N_{max}). The minimum SSE was determined using the *lsqnonlin* routine of the Optimization Toolbox of Matlab (The Mathworks Inc.). Simultaneous with parameter estimation, the parameter estimation errors were determined based on the Jacobian matrix.

2.7 Statistical analysis

Analysis of variance (ANOVA) test was performed to determine whether there are 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 AND DISCUSSION

In Figures 1 and 2, the growth curves for planktonic cells and surface colonies of *S. Typhimurium* and *L. monocytogenes* are presented for all experimental conditions. Growth curves are fitted with the Baranyi and Roberts (1994) model. For *S. Typhimurium* at pH 6.0 and 8% (w/v) NaCl in a solid environment, growth was not observed. Table 2 summarizes the estimated main growth parameters from the Baranyi and Roberts model, i.e., lag time λ [h], maximum specific growth rate μ_{max} [1/h], maximum cell density N_{max} [ln(CFU/mL)] and corresponding statistical analyses.

3.1 Influence of growth morphology on the growth dynamics of *S. Typhimurium* and *L. monocytogenes*

First of all, the influence of the growth morphology, resulting from the intrinsic food (model) structure, on the growth behavior is studied for *S. Typhimurium* and *L. monocytogenes*. When comparing growth curves for planktonic cells and surface colonies of both microorganisms, at most experimental conditions no clear differences are present. For *S. Typhimurium* at most experimental conditions, growth curves of planktonic cells and surface colonies are rather similar although surface colonies often appear to have slightly higher growth rates and reach higher maximum cell densities. When conditions become more extreme due to higher salt concentrations or low pH values, this trend seems to be reversed (pH 5.5, 6% (w/v) NaCl). At the most extreme condition (pH 6.0, 8% (w/v) NaCl), only planktonic cells were able to grow. For *L. monocytogenes*, similar growth dynamics are detected for both growth morphologies at most experimental conditions. Differences in growth behavior between the growth morphologies become more evident for experimental conditions with an increased salt level. Unlike *S. Typhimurium*, both growth morphologies of *L. monocytogenes* are able to grow at very high salt concentrations.

These trends in the growth curves when comparing the different growth morphologies are also confirmed by the estimated growth parameters and the statistical analysis. For *S. Typhimurium*, results of parameter estimation indicate that the maximum specific growth rate μ_{max} under most conditions is significantly higher for surface colonies. At pH 5.5 and 2% (w/v) NaCl, statistical analysis shows no differences between the two growth morphologies, but still the μ_{max} value is slightly higher for surface colonies. Exceptions to this trend are observed at pH 5.5 and 6% (w/v) NaCl where planktonic cells have a significantly higher μ_{max} , and at the most severe condition (pH 6.0, 8% (w/v) NaCl), where surface colonies are no longer able to grow. Similar to μ_{max} , the maximum cell density reached for the surface colonies is slightly higher or equal to the N_{max} for planktonic cells, for most experimental conditions. Almost no significant differences are observed for the duration of the lag phase for the two growth morphologies.

For *L. monocytogenes*, different trends as compared to *S. Typhimurium* are observed in the growth parameter estimates (μ_{max} , lag , N_{max}). μ_{max} estimates of planktonic cells and surface colonies are similar for most conditions, except when either no salt or high concentrations of salt are present in the growth medium. The trend observed for *S. Typhimurium*, where surface colonies exhibit a higher μ_{max} than planktonic cells, also applies for *L. monocytogenes* when no salt is added to the growth medium. However, at the highest salt levels, this trend is even reversed and planktonic cells have a higher μ_{max} . For most conditions, N_{max} values are similar for the two growth morphologies, except at high salt concentrations, where surface colonies tend to have the highest maximum cell density. At more stressing conditions with high salt levels, planktonic cells have longer lag phases, while the lag time duration is rather similar for both growth morphologies.

Different trends in growth behavior are observed for both microorganisms. This divergence in results is due to differences in the structure of the cell wall for both microorganisms. *S. Typhimurium* is a gram-negative bacteria and has a very thin peptidoglycan layer sandwiched between an inner cell membrane and a bacterial outer membrane. *L. monocytogenes* however, gram-positive, has a very thick peptidoglycan layer but no outer membrane (Salton et al., 1996). In literature, it is often reported that gram-positive bacteria are more resistant to adverse environmental conditions than gram-negative bacteria (Jasson et al., 2007; Yuste et al., 2004). Compared to *S. Typhimurium*, the growth range of *L. monocytogenes* is wider and the microorganism is able to grow under harsh conditions, e.g., at refrigerator temperatures (psychotropic microorganism). These facts also explain why in the most severe condition (pH 6.0, 8% (w/v) NaCl), surface colonies are only able to grow for experiments performed with *L. monocytogenes*.

For *S. Typhimurium* under most experimental conditions, surface colonies tend to have higher μ_{max} values than planktonic cells. For *L. monocytogenes*, this trend is also observed when no salt is added. However, for most other experimental conditions, no significant differences in μ_{max} between both growth morphologies are present. In most literature, the opposite behavior as mentioned above is described, with planktonic cells having higher maximum specific growth rates than (surface) colonies

(Aspridou et al., 2014; Brocklehurst et al., 1997; Meldrum et al., 2003; Theys et al., 2008). Literature describes that adding gelling agents to the system, inducing colony growth, results in a lower μ_{max} compared to planktonic growth. However, in these studies the liquid system is constantly shaken ensuring a homogenized system and consequently a constant oxygen supply. In the present work, it was chosen to perform both liquid and solid(like) experiments in small petri dishes under static conditions to isolate the effect of the growth morphology and to create a more realistic scenario for food products. This static set-up is similar to experiments in Smet et al. (2015), where the effect of suboptimal temperatures is studied on the growth dynamics of planktonic cells and colonies of *S. Typhimurium* and *E. coli* and similar growth parameters were observed for planktonic cells, immersed colonies and surface colonies. For planktonic cells, static incubation results in cell sedimentation and lower oxygen concentrations as compared to shaken experimental conditions. For static incubation, oxygen availability for planktonic cells is reduced as compared to the oxygen concentration for surface colonies, which are directly exposed to the atmospheric oxygen. Consequently, planktonic cells have lower or similar μ_{max} values as surface colonies, as observed in the present work for both microorganisms. These results indicate that under certain experimental growth conditions, the effect of the microstructure becomes less pronounced or even negligible as compared to the oxygen availability in the system.

In order to study the effect of the static incubation on the growth dynamics of planktonic cells of *S. Typhimurium* and *L. monocytogenes*, an additional experiment at pH 6.5 and 2% (w/v) NaCl was performed by shaking the petri dishes during incubation. The results, together with those from the static liquid and solid(like) systems are summarized in Figure 3 and Table 3. For both microorganisms, a good oxygen availability (due to the shaken conditions) stimulate microbial growth, and higher values for μ_{max} and N_{max} are estimated. The lag phase is less affected by the shaking, with similar or slightly lower values for the lag duration as compared to the static systems. These results confirm the important role of oxygen and its influence on microbial growth. The additional experiments with shaken liquid cultures explain why under static incubation, for both microorganisms and most

experimental conditions, the order $\mu_{max, planktonic\ cells} \leq \mu_{max, surface\ colonies}$ is followed, which is in contrast with most literature.

However, at very high salt concentrations for both microorganisms, planktonic cells have higher μ_{max} than the surface colonies. This is in line with literature (e.g., Antwi et al., 2006; Aspridou et al., 2014; Theys et al., 2008) and indicates that for very stressing conditions the microstructure does become decisive. By combining stress factors, in this case a high salt concentration and a solid environment, growth is reduced due to a synergistic inhibitory effect known as the hurdle technology (Leistner, 2000). The addition of NaCl to the growth medium induces a decrease in a_w , influencing microbial growth. This effect, independent of the growth morphology, is extensively discussed in Section 3.2. The presence of a structuring agent to the growth medium causes an extra decrease in a_w values. However, as can be deduced from Table 1, this extra decrease is only very pronounced at very high salt concentrations, leading to large differences in a_w between planktonic cells and surface colonies. For the most stressing experimental conditions, this large difference in a_w explains the order $\mu_{max, planktonic\ cells} > \mu_{max, surface\ colonies}$.

3.2 Osmotolerance of planktonic cells and surface colonies of *S. Typhimurium* and *L. monocytogenes*

In order to investigate the influence of salt on the growth dynamics of planktonic cells and surface colonies, the variation of growth parameter estimates as a function of the salt concentration is presented in Figure 4 for *S. Typhimurium* and Figure 5 for *L. monocytogenes*.

By increasing the salt level, the growth environment becomes more stressing for the cells, and consequently, μ_{max} and N_{max} decrease, as often reported in literature. For planktonic cells of *S. Typhimurium*, this decrease is very clear over the complete range of salt concentrations at all pH values. For the surface colonies, a decrease for both parameters is noticeable over the range [0-6 % (w/v) NaCl], leading to absence of growth at pH 6.0 and 8% (w/v) NaCl. For high salt concentrations, a

significant elongation of the lag time has been noted for both growth morphologies. The above mentioned trends for *S. Typhimurium* are even more pronounced when comparing, for the same growth morphology, experimental conditions sharing the same pH and just varying in salt concentration. For the same pH value, *S. Typhimurium* cells of both growth morphologies have the longest lag phase for the condition with the highest salt level. At the same time, for the condition with the highest salt level, cells also have a lower maximum specific growth rate and maximum cell density.

An elongation of the lag phase, combined with a decrease of μ_{max} and N_{max} when the salt level increases, is also detected for *L. monocytogenes*, although less pronounced as for *S. Typhimurium*. Especially when studying surface colonies, almost no increase of the lag time as a function of salt concentration is noticeable, whereas for planktonic cells the increase in lag duration due to the addition of salt is very pronounced. For μ_{max} , the expected decrease with an increasing salt level is observed for both growth morphologies. When focusing on either planktonic cells or surface colonies at the same pH value, again this decrease in μ_{max} with an increasing salt concentration is very pronounced. Considering the maximum cell density for both planktonic cells and surface colonies, no clear trend can be deducted for *L. monocytogenes*.

Similar results were reported in Boons et al. (2013) for both *S. Typhimurium* and *E. coli*. With an increasing salt concentration the lag phase elongates while both μ_{max} and N_{max} decrease. Adding salt to the growth medium has a direct effect on the water activity; a low a_w value can exert a severe stress on the microorganisms and eventually lead to their death (O'Byrne and Booth, 2002). The minimal a_w values required for growth are 0.940 for *S. Typhimurium* and 0.920 for *L. monocytogenes* (adapted using NaCl, ICMSF, 1996). Water activity values for all systems were summarized in Table 1. In the liquid system, the addition of 8% (w/v) NaCl decreased the water activity to 0.948 in TSB (*S. Typhimurium*) and 0.939 in BHI (*L. monocytogenes*). These values are still above the growth limits, explaining that planktonic growth is still detected, although it might be seriously slowed down. In the gelatinized system, when adding 8% (w/v) salt, a_w values drop to 0.933 (*S. Typhimurium*) and 0.937

(*L. monocytogenes*), due to the combined effect of salt and gelation addition. The a_w value for TSB, supplemented with 5% (w/v) gelatin, at pH 6.0 and 8% (w/v) NaCl is below the growth limit for *S. Typhimurium*, explaining the absence of colonial growth.

3.3 Acid tolerance of planktonic cells and surface colonies of *S. Typhimurium* and *L. monocytogenes*

Next to the growth morphology, as a consequence of the intrinsic food (model) structure, and the osmotic stress, exposure to an acidic environment also affects the microbial growth dynamics. However, since pH values for all experimental conditions are mild as compared to the selected range of salt concentrations, the influence of the pH on the microbial growth dynamics of *S. Typhimurium* and *L. monocytogenes* is less obvious than the influence of the salt concentration. Creating a slightly more stressed environment by the addition of acid to the growth medium, does not seem to have an effect on the growth parameters, independently of the effect caused by salt concentration and growth morphology (Table 2). Only by comparing the results at varying pH values, for the same microorganism, growth morphology and salt level, some trends can be deducted (Figure 5, 6 and Table 2). For *L. monocytogenes*, conditions with a more optimal pH have higher μ_{max} and N_{max} but shorter lag times. This trend is noticeable for both growth morphologies. For *S. Typhimurium*, parameters values for varying pH values at a certain salt level and growth morphology are rather similar in most cases. Significant differences are observed for both growth morphologies at 6% (w/v) NaCl, with shorter lag phases but higher values for μ_{max} and N_{max} at pH 5.5 than at the more optimal pH 6.5.

As mentioned before, pH values in this research in the range [5.5-7.0] are mild and far from the growth limits. For *S. Typhimurium*, the range for growth is between pH of 3.8 and 9.5, with the optimum pH for growth around 7.0-7.5. A similar growth range is deducted for *L. monocytogenes*,

with a pH_{min} of 4.39, a pH_{opt} of 7.0 and a pH_{max} of 9.4 (ICMSF, 1996). This explains the minimal effect of the pH on the growth behavior of planktonic cells and surface colonies for both microorganisms.

Different intrinsic factors have an effect on the growth behavior of *S. Typhimurium* and *L. monocytogenes*. Whereas the influence of the pH at the selected conditions turns out to be negligible, the amount of salt added to the system proved to be important. For the conditions selected, the growth morphology, as a consequence of the growth medium structure, has an important influence on the growth behavior. In contrast to most literature, where planktonic cells exhibit the highest μ_{max} , in this work surface colonies tend to have higher or similar maximum specific growth rate, which is attributed to the static conditions selected. Static conditions lead to lower oxygen availability in the liquid system, as compared to the shaken systems in literature, and expected is a more similar oxygen availability comparable to solid systems. This explains the observed trends for both growth morphologies and indicates that under certain static experimental conditions, oxygen availability plays a more decisive role than the microstructure. However at very stressing conditions, e.g., when the salt concentration is very high, colony growth is reduced, as compared to planktonic growth, due to the hurdle technology. This study indicates the relevance of intrinsic food factors on the growth dynamics of foodborne pathogens of concern and highlights the importance to include the factor food structure, and consequently the growth morphology, in predictive models.

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FIGURES

Figure 1: Growth dynamics of *S. Typhimurium* for both planktonic cells and surface colonies. The data are fitted with the model of Baranyi and Roberts. a) pH 7.0, 0% (w/v) NaCl; b) pH 6.0, 0% (w/v) NaCl; c) pH 6.5, 2% (w/v) NaCl; d) pH 5.5, 2% (w/v) NaCl; e) pH 7.0, 4% (w/v) NaCl; f) pH 6.0, 4% (w/v) NaCl; g) pH 5.0, 4% (w/v) NaCl; h) pH 6.5, 6% (w/v) NaCl

Figure 1 (continued): Growth dynamics of *S. Typhimurium* for both planktonic cells and surface colonies. The data are fitted with the model of Baranyi and Roberts. i) pH 5.5, 6% (w/v) NaCl; j) pH 6.0, 8% (w/v) NaCl

Figure 2: Growth dynamics of *L. monocytogenes* for both planktonic cells and surface colonies. The data are fitted with the model of Baranyi and Roberts. a) pH 7.0, 0% (w/v)

NaCl; b) pH 6.0, 0% (w/v) NaCl; c) pH 6.5, 2% (w/v) NaCl; d) pH 5.5, 2% (w/v) NaCl; e) pH 7.0, 4% (w/v) NaCl; f) pH 6.0, 4% (w/v) NaCl; g) pH 5.0, 4% (w/v) NaCl; h) pH 6.5, 6% (w/v) NaCl

Figure 2 (continued): Growth dynamics of *L. monocytogenes* for both planktonic cells and surface colonies. The data are fitted with the model of Baranyi and Roberts. i) pH 5.5, 6% (w/v) NaCl; j) pH 6.0, 8% (w/v) NaCl

Figure 3: Comparison of the growth dynamics of planktonic cells (shaken), planktonic cells (static) and surface colonies of *S. Typhimurium* (left) and *L. monocytogenes* (right) at pH 6.5 and 2% (w/v) NaCl. The data are fitted with the model of Baranyi and Roberts.

Figure 4: Growth parameters of *S. Typhimurium* as a function of the salt concentration for both planktonic cells (left) and surface colonies (right), at pH 5.0, pH 5.5, pH 6.0, pH 6.5 and pH 7.0.

Figure 5: Growth parameters of *L. monocytogenes* as a function of the salt concentration for both planktonic cells (left) and surface colonies (right), at pH 5.0, pH 5.5, pH 6.0, pH 6.5 and pH 7.0.

TABLES

Table 1: Water activities of the media supporting planktonic cells and surface colonies of *S. Typhimurium* and *L. monocytogenes*.

Reference	NaCl (% (w/v))	pH (-)	a_w of growth media			
			<i>Salmonella</i> Typhimurium		<i>Listeria</i> <i>monocytogenes</i>	
			Planktonic cells	Surface colonies	Planktonic cells	Surface colonies
a	0	7.0	0.994	0.994	0.994	0.991
b		6.0	0.985	0.991	0.990	0.990
c	2	6.5	0.973	0.979	0.979	0.978
d		5.5	0.975	0.975	0.979	0.978
e	4	7.0	0.972	0.966	0.969	0.965
f		6.0	0.969	0.958	0.967	0.966
g		5.0	0.966	0.959	0.966	0.963
h	6	6.5	0.959	0.946	0.956	0.949
i		5.5	0.955	0.951	0.959	0.950
j	8	6.0	0.948	0.933	0.939	0.937

Table 2. Kinetic parameters of the Baranyi and Roberts model obtained for planktonic cells and surface colonies of *S. Typhimurium* and *L. monocytogenes* at the different growth conditions.

Strain	Condition			Kinetic parameters							
	NaCl (% (w/v))	pH (-)	Reference	1Lag (h)^2_3		$1\mu_{\max} (1/h)^2_3$		$1N_{\max} (\text{CFU/mL})^2_3$		RMSE	
				Planktonic cells	Surface colonies	Planktonic cells	Surface colonies	Planktonic cells	Surface colonies	Planktonic cells	Surface colonies
<i>Salmonella Typhimurium</i>	0	7.0	a	(a)0.9 ± 0.6 ^a _A	(a)1.0 ± 0.6 ^a _A	(a)0.457 ± 0.009 ^a _B	(b)0.505 ± 0.010 ^a _B	(a)21.3 ± 0.1 ^a _B	(b)21.7 ± 0.1 ^a _B	0.304	0.317
		6.0	b	(a)1.9 ± 0.3 ^a _A	(a)2.0 ± 0.7 ^a _A	(a)0.475 ± 0.006 ^b _C	(b)0.530 ± 0.014 ^a _B	(a)21.4 ± 0.1 ^a _C	(b)22.1 ± 0.1 ^b _B	0.190	0.310
	2	6.5	c	(a)3.3 ± 0.5 ^a _A	(a)3.7 ± 0.9 ^a _A	(a)0.435 ± 0.008 ^a _B	(b)0.464 ± 0.011 ^a _B	(a)20.9 ± 0.1 ^b _B	(a)20.9 ± 0.1 ^a _B	0.163	0.236
		5.5	d	(a)3.7 ± 0.9 ^a _A	(a)4.4 ± 0.6 ^a _A	(a)0.433 ± 0.016 ^a _B	(a)0.449 ± 0.009 ^a _B	(a)20.5 ± 0.1 ^a _B	(b)21.4 ± 0.1 ^b _B	0.297	0.220
	4	7.0	e	(a)14.3 ± 1.2 ^b _B	(a)12.6 ± 0.7 ^b _B	(a)0.233 ± 0.008 ^a _B	(b)0.260 ± 0.006 ^a _A	(a)20.2 ± 0.2 ^a _B	(a)20.4 ± 0.1 ^c _A	0.312	0.227
		6.0	f	(a)10.1 ± 0.6 ^a _A	(b)12.5 ± 0.8 ^b _B	(a)0.260 ± 0.005 ^c _B	(b)0.297 ± 0.008 ^a _B	(b)20.1 ± 0.1 ^b _B	(a)19.8 ± 0.1 ^a _A	0.177	0.214
	5	5.0	g	(a)10.6 ± 0.9 ^a _A	(a)10.0 ± 1.0 ^a _A	(a)0.220 ± 0.005 ^a _A	(b)0.249 ± 0.007 ^a _A	(a)19.8 ± 0.1 ^a _A	(b)20.2 ± 0.1 ^b _B	0.203	0.202
		6.5	h	(b)43.8 ± 2.5 ^b _B	(a)37.2 ± 1.4 ^b _B	(a)0.127 ± 0.004 ^a _A	(b)0.182 ± 0.006 ^b _A	(a)18.8 ± 0.1 ^a _A	(b)19.4 ± 0.1 ^a _A	0.326	0.356
	5.5	5.5	i	(a)22.9 ± 1.8 ^a _B	(a)19.4 ± 4.0 ^a _B	(b)0.183 ± 0.010 ^b _A	(a)0.142 ± 0.010 ^a _A	(a)19.4 ± 0.1 ^b _A	(b)20.2 ± 0.2 ^b _A	0.279	0.439
		6.0	j	70.0 ± 14.0 _B	no growth	0.038 ± 0.002 _A	no growth	18.0 ± 0.3 _A	no growth	0.841	no growth
<i>Listeria monocytogenes</i>	0	7.0	a	(a)1.0 ± 0.5 ^a _A	(b)3.0 ± 0.7 ^a _A	(a)0.435 ± 0.008 ^b _B	(b)0.473 ± 0.012 ^b _B	(a)21.1 ± 0.1 ^b _A	(b)21.7 ± 0.1 ^b _B	0.240	0.319
		6.0	b	(a)0.1 ± 0.6 ^a _A	(b)3.9 ± 1.0 ^a _A	(a)0.375 ± 0.007 ^a _C	(b)0.424 ± 0.016 ^a _C	(a)20.8 ± 0.1 ^a _B	(a)20.6 ± 0.2 ^a _A	0.200	0.403
	2	6.5	c	(a)1.4 ± 0.8 ^a _A	(a)2.8 ± 1.2 ^a _A	(a)0.374 ± 0.008 ^b _B	(a)0.365 ± 0.013 ^a _B	(a)21.0 ± 0.1 ^b _A	(a)21.0 ± 0.1 ^b _A	0.250	0.361
		5.5	d	(a)6.7 ± 1.0 ^b _A	(a)9.7 ± 3.0 ^b _A	(a)0.338 ± 0.015 ^a _B	(a)0.319 ± 0.046 ^a _B	(a)20.5 ± 0.1 ^a _B	(a)20.1 ± 0.4 ^a _A	0.221	0.672
	4	7.0	e	(a)4.8 ± 0.9 ^a _B	(a)5.7 ± 1.3 ^b _B	(a)0.326 ± 0.011 ^c _A	(a)0.341 ± 0.019 ^c _A	(a)21.1 ± 0.1 ^c _A	(a)21.3 ± 0.1 ^c _A	0.189	0.300
		6.0	f	(a)3.8 ± 0.9 ^a _B	(a)1.4 ± 1.7 ^a _A	(b)0.288 ± 0.008 ^b _B	(a)0.264 ± 0.011 ^b _B	(a)20.9 ± 0.1 ^b _B	(a)21.0 ± 0.2 ^b _B	0.211	0.419
	5	5.0	g	(a)30.6 ± 1.5 ^b _B	(b)51.9 ± 2.5 ^c _C	(a)0.154 ± 0.005 ^a _A	(b)0.209 ± 0.012 ^a _A	(a)18.8 ± 0.1 ^a _A	(b)20.3 ± 0.1 ^a _A	0.333	0.680
		6.5	h	(b)7.4 ± 0.7 ^a _B	(a)2.7 ± 1.7 ^a _A	(b)0.248 ± 0.005 ^b _A	(a)0.204 ± 0.006 ^a _A	(a)20.9 ± 0.0 ^b _A	(b)21.2 ± 0.1 ^b _A	0.146	0.278
	5.5	5.5	i	(b)18.6 ± 3.2 ^b _B	(a)9.1 ± 4.3 ^a _A	(a)0.166 ± 0.011 ^a _A	(a)0.185 ± 0.014 ^a _A	(a)18.6 ± 0.1 ^a _A	(b)20.7 ± 0.2 ^a _A	0.529	0.755
		6.0	j	(b)29.4 ± 2.9 _C	(a)16.8 ± 2.4 _B	(b)0.145 ± 0.008 _A	(a)0.113 ± 0.003 _A	(a)19.3 ± 0.1 _A	(b)20.5 ± 0.1 _A	0.430	0.329

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

² For each individual microorganism, salt level (0-2-4-6-8 % (w/v) NaCl) and growth morphology, parameters of the Baranyi and Roberts model bearing different superscripts (no lowercase letters in common) are significantly different ($P \leq 0.05$)

³ For each individual microorganism, pH value (5.5-5.5-6.0-6.5-7.0) and growth morphology, parameters of the Baranyi and Roberts model bearing different subscripts (no uppercase letters in common) are significantly different ($P \leq 0.05$)

Table 3. Kinetic parameters of the Baranyi and Roberts model obtained for planktonic cells (shaken), planktonic cells (static) and surface colonies of *S. Typhimurium* and *L. monocytogenes* at pH 6.5 and 2% (w/v) NaCl.

Strain	Growth morphology	Conditions	Kinetic parameters			
			μ_{Lag} (h)	μ_{max} (1/h)	N_{max} (CFU/mL)	RMSE
<i>Salmonella</i> <i>Typhimurium</i>	Planktonic cells	Shaken	$^{(a)}2.0 \pm 0.6$	$^{(b)}0.655 \pm 0.022$	$^{(b)}21.3 \pm 0.1$	0.351
		Static	$^{(ab)}3.3 \pm 0.5$	$^{(a)}0.435 \pm 0.008$	$^{(a)}20.9 \pm 0.1$	0.163
	Surface colonies	Static	$^{(b)}3.7 \pm 0.9^b$	$^{(a)}0.464 \pm 0.011$	$^{(a)}20.9 \pm 0.1$	0.236
<i>Listeria</i> <i>monocytogenes</i>	Planktonic cells	Shaken	$^{(a)}1.5 \pm 0.7$	$^{(b)}0.595 \pm 0.019$	$^{(b)}21.5 \pm 0.1$	0.357
		Static	$^{(a)}1.4 \pm 0.8$	$^{(a)}0.374 \pm 0.008$	$^{(a)}21.0 \pm 0.1$	0.250
	Surface colonies	Static	$^{(a)}2.8 \pm 1.2$	$^{(a)}0.365 \pm 0.013$	$^{(a)}21.0 \pm 0.1$	0.361

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