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Different carbon sources result in differential activation of sigma B and stress resistance in *Listeria monocytogenes*

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Abstract

Listeria monocytogenes is an important food-borne pathogen that is ubiquitous in the environment. It is able to utilise a variety of carbon sources, to produce biofilms on food-processing surfaces and to survive food preservation–associated stresses. In this study, we investigated the effect of three common carbon sources, namely glucose, glycerol and lactose, on growth and activation of the general stress response Sigma factor, SigB, and corresponding phenotypes including stress resistance. A fluorescent reporter coupled to the promoter of *lmo2230,* a highly SigB-dependent gene, was used to determine SigB activation via quantitative fluorescence spectroscopy. This approach, combined with Western blotting and fluorescence microscopy, showed the highest SigB activation in lactose grown cells and lowest in glucose grown cells. In line with this observation, lactose grown cells showed the highest resistance to lethal heat and acid stress, the highest biofilm formation, and had the highest adhesion/invasion capacity in Caco-2-derived C2Bbe1 cell lines. Our data suggest that lactose utilisation triggers a strong SigB dependent stress response and this may have implications for the resistance of *L. monocytogenes* along the food chain.

1. Introduction

Listeria monocytogenes is a Gram positive rod that is found ubiquitously in the environment. Common environmental niches of *L. monocytogenes* include soil, plants, faecal matter and animal fodder (Tiensuu et al., 2019; Weis and Seeliger, 1975). In the environment, the risk of *L. monocytogenes* infecting humans is low, but its transfer into the food chain, particularly to foods that require no cooking prior to consumption, can result in life-threatening listeriosis (Farber and Peterkin, 1991). To survive in such a variety of niches, ranging from soil to the human gastrointestinal tract, *L. monocytogenes* must be able to adapt efficiently to a variety of environmental conditions and be capable of utilising a diverse range of nutrient sources (Sauer et al., 2019).

In the different environments in which *L. monocytogenes* can survive, the availability of nutrients varies greatly, and therefore, this microorganism must be able to utilize different compounds as carbon and/or energy sources in order to grow in the potential niches it can occupy. In order to cope with these changing conditions, *L. monocytogenes* is equipped with a diverse range of transport systems for different sugars. The genome of this bacterium contains a significant number of ABC transporters and phosphoenolpyruvate: sugar phosphotransferase (PTS) systems linked to the transport of sugars, most of which have not yet been fully characterised (Deutscher et al., 2014; Stoll and Goebel, 2010).

Common laboratory culture media used for the growth of *L. monocytogenes* include glucose as the main, if not sole, carbon source. Studies investigating the effects of glucose metabolism on gene transcription have shown that it inhibits PrfA activity, the main

regulator of virulence in *L. monocytogenes* (Behari and Youngman, 1998). A similar study investigating the effects of glycerol metabolism on PrfA activity suggested that glycerol metabolism activates PrfA, thereby increasing virulence (Joseph et al., 2008). Moreover, a mutant of *L. monocytogenes* strain 10403S lacking Sigma B (Δ*sigB*) showed reduced growth performance with glycerol as the main carbon source in a chemically defined medium (Abram et al., 2008). As these studies show, the carbon sources that *L. monocytogenes* is able to metabolise may alter gene transcription and regulation, potentially influencing its ability to survive food processing stresses and infect a human host.

Metabolism of glucose and glycerol has been relatively well studied in *L. monocytogenes*, whereas the metabolism of other carbon and energy sources and impact on performance has gained less attention. It is known that *L. monocytogenes* can grow with simple sugars such as mannose and fructose, and more complex molecules like cellobiose and lactose (Stoll and Goebel, 2010). Lactose is a disaccharide composed of one glucose molecule linked to one galactose molecule by a β-1,4-glycosidic bond. Lactose is the main carbohydrate in milk and as such, is likely to be encountered by *L. monocytogenes* in food processing environments involving dairy products. Notably, listeriosis outbreaks have repeatedly been linked to the consumption of dairy products (Castro et al., 2018; Kim et al., 2018; Sauders and D'Amico, 2016).

Previous studies have shown that most *L. monocytogenes* strains are able to utilize lactose as a carbon source to a certain degree (Pine et al., 1989), but little is known about the metabolic pathway or the genes involved. It has been reported that *L. monocytogenes* uses only the glucose moiety of the lactose molecule, and exports the galactose moiety out of the

cell, but the mechanism by which this happens remains to be elucidated (Pine et al., 1989). A more recent paper by (Dalet et al., 2003) describes a set of genes that might be involved in the transport of the lactose molecule inside the cell, but the results were not conclusive. The genes belonging to the *lpo* operon were upregulated during growth in a lactose-based media; however, deletion mutants lacking some of the operon elements were still able to grow with lactose (Dalet et al., 2003), suggesting that alternative pathways might play a role in lactose metabolism in *L. monocytogenes.*

The impact of lactose metabolism on *L. monocytogenes* stress resistance and/or virulence has not yet been investigated. The stress response regulator SigB is one of the key players in the regulation of the general stress response of *L. monocytogenes* controlling the expression of a large number of stress defence proteins as described previously and in recent reviews (Becker et al., 1998; Dorey et al., 2019; Ferreira et al., 2001; Guldimann et al., 2016; NicAogain and O'Byrne, 2016; Sue et al., 2004). In addition, a *L. monocytogenes ΔsigB* deletion mutant showed a reduced ability to survive under carbon limiting conditions, suggesting that SigB might also be involved in resistance to prolonged starvation (Ferreira et al., 2001; Herbert and Foster, 2001).

Based on the role of *L. monocytogenes* SigB in substrate utilisation, stress defence, and virulence, we decided to perform a comparative analysis of the impact of glucose, glycerol and lactose metabolism on *L. monocytogenes* EGDe activation of SigB in exponential and stationary phase cells and to determine the effect on acid and heat resistance, biofilm formation and Caco-2 cell adhesion and invasion. Our data highlight no apparent role of SigB in *L. monocytogenes* lactose utilisation and growth, but provide evidence for lactose-induced

SigB-dependent stress defences that could contribute to its transmission in the food chain, especially those foods containing lactose.

2. Material and Methods

2.1 Bacterial strains and growth conditions

In this study *L. monocytogenes* strain EGDe wildtype (WT), its *ΔsigB* deletion mutant, and an EGDe strain with the plasmid pKSV7::P_{2230}-egfp integrated in the chromosome (Utratna et al., 2012) were used. The presence of the enhanced green fluorescence protein gene (*egfp*) in this plasmid allowed for the monitoring of SigB activity as the promoter of *lmo2230* is highly SigB-dependent (Utratna et al., 2012). Permanent stocks of each strain were stored in brain heart infusion (BHI) (Becton Dickinson Difco) broth supplemented with 15% (v/v) glycerol (Fluka) at -80°C. Permanent stocks were streaked onto BHI agar (Becton Dickinson Difco), incubated at 30°C overnight then stored at 4°C for up to 1 month. For overnight cultures, a single colony was taken from an agar plate, inoculated into BHI broth and incubated overnight at 30°C, shaking at 160 rpm. Unless otherwise specified, experiments were carried out in nutrient broth (NB, Oxoid) supplemented to 0.4% (w/v) with glucose (Sigma-Aldrich), glycerol or lactose (VWR Chemicals), using autoclaved stocks of 25% (w/v) glucose, glycerol and lactose prepared in deionised water. Phosphate buffered saline (PBS) was prepared by dissolving 8.98 g Na_2HPO_4 (Merck), 2.72 g $NaH_2PO_4 \cdot H_2O$ (Merck) and 8.5 g NaCl (Sigma-Aldrich) in 1 L deionised H_2O.

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2.2 Planktonic growth curves

Overnight cultures of the wild-type and Δ*sigB* deletion mutant strains were diluted to OD_{600} 0.1 in each of the culture media (NB supplemented with glucose, glycerol or lactose), and 350 µl aliquots were made in triplicate into 100-well Honeycomb® microplates (ThermoFisher). Plates were incubated at 30°C for 48 h using the Bioscreen C (ThermoFisher) with continuous shaking at medium intensity, and OD_{600} was measured at 2 h intervals. A blank control was included and subtracted from the raw values. The experiment was performed with two biologically independent replicates, with three technical replicates each.

2.3 Quantitative fluorescence spectroscopy

The overnight culture of the eGFP reporter strain was diluted in supplemented NB to an OD_{600} 0.01 and incubated in 48-well plates (Greiner bio), shaking, with a final volume of 1 mL per well, at 30^{o}C in darkness. At selected time points, OD_{600} and fluorescence intensity (ex/em 480/530) were measured using the Spectramax M2 plate reader (Molecular Devices). A blank control was included for each culture medium, and its fluorescence intensity values were subtracted from the tested conditions. To correct for background fluorescence generated by increasing cell density, a standard curve was prepared using EGDe wild-type cells without the integrated eGFP reporter in each of the culture mediums. The OD_{600} and fluorescence was measured for each sample of the standard curve, and the background fluorescence for each test sample, interpolated from the standard curve based on the OD_{600} of the sample, was subtracted from the fluorescence reading. The experiment was performed with two biologically independent replicates, with three technical replicates each.

2.4 Protein sample collection and extraction

The overnight culture of the eGFP reporter strain was diluted in supplemented NB to an OD_{600} 0.01, and incubated at 30°C, shaking, for 24 h in darkness. A 100 ml aliquot was taken from each culture, and 10 µg/mL of chloramphenicol was added before protein extraction to stop protein translation. Samples were centrifuged for 15 min at 9000 *x g* at 4°C. The bacterial pellet was resuspended in 2 mL sonication buffer (10 mM Tris-HCl (Sigma-aldrich), 0.1 mM EDTA (AnalaR), 5 mM $MgCl_2$ (AnalaR), adjusted to pH 8 and autoclaved) supplemented with 2 mg/ml lysozyme (Sigma-Aldrich) and incubated for 30 min at 37°C, shaking. The culture was centrifuged 9000 *x g* for 15 min at 4°C and the pellet was resuspended in 0.5 ml of sonication buffer supplemented with 1% (v/v) protease inhibitor (Sigma-Aldrich). Cells were lysed by bead beating for two 40 s cycles at 6 m/s in FastPrep® 1 ml Matrix B lysis tubes (MP Biomedicals) with a FastPrep®-24 *Classic* Instrument (MP Biomedicals). The preparation was centrifuged 13000 *x g* for 30 min at 4°C to remove cell debris. Protein quantification was carried out using the *DC*™ Protein Assay (BioRad) according to manufacturers' instructions.

2.5 Western blot analysis

The protein content of each sample was equalised to 0.8 mg/mL and 20 µl of each sample was separated by SDS-PAGE and transferred to a PVDF membrane. Western blot analysis was performed using rabbit anti-eGFP primary antibody (Santa Cruz Biotechnology), diluted 1:7500 in 5% skim milk and incubated for 16 h at 4°C. The membrane was incubated for 1 h in mouse anti-rabbit secondary antibody (Santa Cruz Biotechnology) diluted 1:3500 in 5%

skim milk. Blots were imaged using a chemiluminescent substrate (Amersham) on a LICOR Odyssey® Fc Imaging System (LI-COR Biosciences). Image Studio (LI-COR Biosciences) was used to process and analyse the images.

2.6 Fluorescence microscopy

The overnight culture of the eGFP reporter strain was diluted in NB with supplements to an OD_{600} of 0.01 and incubated in flasks shaking (160 rpm) at 30°C, in darkness. Samples for microscopy were taken after 24 hours of incubation, and prepared as described previously (Utratna et al., 2012). Briefly, 1 mL aliquots of the culture were centrifuged and washed twice in equal volume of PBS. Subsequently the pellet was resuspended in PBS to an OD_{600} of 1.0, to obtain similar concentrations of cells per sample, and observed under the microscope (Olympus BX41), 100X. For each sample, phase contrast and the eGFP fluorescence (ex/em 480/530) images were taken with the CellB software. Representative images of each slide were taken. The experiment was performed with two biologically independent replicates.

2.7 Heat resistance

The overnight culture of the wild type EGDe strain was diluted in NB supplemented with glucose, glycerol or lactose to an OD_{600} of 0.01, and incubated in flasks shaking (160 rpm) at 30°C. The heat inactivation assay was performed at 55°C in a water bath with shaking at 160 rpm (Julabo SW 23, Julabo Labortechnik) as described before (Metselaar et al., 2015). Samples were taken after 5 and 24 h of incubation, representing actively growing and stationary phase cultures, respectively. Briefly, 200-400 µL of the culture were inoculated into 20 mL of pre-heated NB with supplements, with an equal final cell concentration. Samples were taken at selected time points up to 45 min, decimally diluted in PBS and spot

(10 µL) plated in triplicate on BHI agar and incubated at 30°C for 48 – 96 h for colony enumeration. The experiment was performed with two biologically independent replicates.

2.8 Acid resistance

The overnight culture of the wild type EGDe strain was diluted in NB with supplements to an OD_{600} of 0.01, and incubated in flasks shaking (160 rpm) at 30^{o}C. The acid inactivation assay was performed at pH 3.0 and pH 2.0 on samples taken after 5 and 24 h of incubation, respectively, in a heat block at 30^{o}C as described before (Metselaar et al., 2013). Briefly, the cultures were centrifuged, washed twice with PBS and resuspended in 2 mL of acidified NB supplemented with the relevant carbon source (pH adjusted with 1 M HCl), to a final concentration of approximately 8 $\log_{10}$ CFU/mL. Samples were taken at selected time points up to 75 minutes, serially diluted in PBS and plated in BHI plates for colony enumeration with the spot plating technique (10 µL per spot), in triplicates. The plates were incubated at 30^{o}C for 2-4 days. The experiment was performed with two biologically independent replicates.

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2.9 Biofilm formation

2.9.1 Crystal Violet staining: polystyrene 96-well plates (Sigma-Aldrich) were inoculated with 1% (v/v) of bacterial overnight culture. Each well was filled with 250 µL of NB with and without supplements. Biofilms were statically incubated for 48 h at 30^{o}C, washed twice with PBS, left to dry for 5 min, and stained with 250 µL of 0.1% crystal violet (CV) for 30 min, as previously described (Crespo Tapia et al., 2018). After staining, the bound CV was dissolved from the biofilm grown in the 96-well plate by adding 70% ethanol and incubation for 15 min, after which the absorbance was measured at 595 nm with the Spectramax M2 plate reader (Molecular Devices). A blank of sterile media was included and subtracted from the experimental values. Experiments were performed with two biologically independent replicates, with three technical replicates each.

2.9.2 Culturable biofilm cells quantification: polystyrene 12-well plates (Sigma-Aldrich) were inoculated with 1% (v/v) of bacterial overnight culture. Each well was filled with 2 mL of NB with and without supplements. Biofilms were statically incubated for 48 hours at 30^{o}C. After incubation, the planktonic cells were aspirated with a pipette, and the plate was washed two times with PBS and dried for a few minutes. After pipetting 1 mL of PBS into individual wells, the biofilm was scrapped from the walls and bottom of each well with a pipette tip and the liquid resuspended thoroughly to avoid clumping of cells, which was checked randomly by microscopy. Appropriate decimal dilutions were prepared in PBS and spiral plated on BHI agar plates, which were incubated at 30°C for 48 hours. Two independent biological replicates and two technical replicates were performed for each condition tested.

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2.10 Virulence assay

C2Bbe1 cells (CRL-2102; American Type Culture Collection), a clone of the Caco2 human adenocarcinoma cell line, were used for the cell invasion assay. Briefly, C2Bbe1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (Gibco), 1% (v/v) penicillin–streptomycin (Gibco), and 0.01 mg/ml human transferrin (Calbiochem) at 37^{o}C in a 5% CO_2 environment. The cells were grown to confluence in tissue culture flasks (Corning Incorporated, NY, USA) following the procedure described previously (Oliveira et al., 2011) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Transfer of the cells to a new flask was performed at 80% confluency. The cell culture medium was replaced every 2–3 days.

To perform the infection assays, C2Bbe1 cells were trypsinized (Gibco), harvested by slow centrifugation at 500 × *g* for 5 min followed by resuspension in antibiotic free, DMEM containing 10% fetal calf serum. Viable cells were counted using a standard trypan blue exclusion procedure. A known concentration of 1 × 10^5 viable cells per well were seeded onto 24-well flat bottom tissue culture plates (Sarstedt, Leicester, United Kingdom). Subsequently, the plates were incubated for 48 h at 37°C in a 5% CO_2 atmosphere until confluent. Prior to the experiment, the cells were washed with PBS once before adding the infection medium (low glucose, antibiotic free DMEM) with the bacterial inoculum.

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Planktonic cultures of *L. monocytogenes* EGDe were grown overnight in BHI medium, re-inoculated into NB with supplements and grown for 24 h. Subsequently, bacterial cells were pelleted, washed once with PBS, resuspended in the infection medium, and added to the C2Bbe1 cells at a multiplicity of infection of 10:1 followed by incubation at 37°C for 2 h in 5% CO_2. Samples for time 0 inoculum quantification were taken from the inoculated infection medium immediately after inoculation of the plates. From here on, the wells for the quantification of adherent and invasive cells, and the quantification of invasive cells only, were treated differently depending on the specific assay:

2.10.1 Adhesion and invasion assay: for the quantification of adherent plus invasive cells, the medium was carefully aspirated and discarded, and the wells were washed 3 times with PBS. 1 ml of 0.1% v/v Triton X-100 (Sigma–Aldrich, Steinheim, Germany) in PBS was added after washing and the plates were incubated for 10 min at room temperature. After that the cells were resuspended and mixed thoroughly, and serially diluted in PBS, appropriate dilutions were spot plated on BHI agar plates and incubated at 30^{o}C for 24-48 h.

2.10.2 Invasion assay: for the quantification of invasive cells alone, gentamicin was added to the media to a final concentration of 50 µg/ml, followed by further incubation of 1 h to kill extracellular bacteria. Subsequently, the C2Bbe1 cells were washed 5x with PBS, treated with Triton X-100 for 10 min, serially diluted in PBS and spot plated on BHI agar plates for enumeration as described before.

Both experiments were performed with two biologically independent replicates, with three technical replicates each.

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2.11 Statistical analysis

The differences in cell counts between the three media in the different stress treatments, biofilm formation and infection assays were analysed by the two-tailed Student's *t*-test. *P* values lower than 0.05 were considered significant. The differences in SigB activity levels were analysed the same way. The test was performed for all the time points of each experiment.

3. Results

3.1 The absence of SigB does not impair growth in any of the tested carbon sources

As the absence of SigB was shown to be detrimental during growth in the presence of glycerol in *L. monocytogenes* 10403S (Abram et al., 2008), we compared the growth of *L. monocytogenes* EGDe wild-type and Δ*sigB* deletion mutant strains in NB supplemented with glucose (Fig. 1A), glycerol (Fig. 1B) or lactose (Fig. 1C).

The WT strain and the Δ*sigB* mutant showed rather similar behaviour in the three carbon sources tested (Fig. 1), as for most of the time points and conditions the OD_{600} values were similar although a slightly lower final OD_{600} was observed for the Δ*sigB* mutant grown in NB-Glycerol (Fig. 1B). We also tested the growth of both WT and mutant strain in non-supplemented NB, and found that a maximum OD_{600} of around 0.13 was reached after 6-8 h of incubation (Fig. S1), whereas with the addition of glucose, glycerol and lactose, cells continued growing for longer times and to higher OD_{600} values.

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3.2 Growth in lactose increases SigB activity

We investigated the activation of the sigma factor SigB during *L. monocytogenes* growth with the different carbon sources, using a genome-integrated SigB-dependent eGFP reporter (Utratna et al., 2012). SigB-dependent eGFP levels were significantly higher in the presence of lactose than in cells grown with glucose from 8 h of incubation until the end of the experiment (Fig. 2A). Cells growing in lactose also showed higher SigB activity compared to cells growing in glycerol at every time point tested, but the differences were only significant after 10 and 12 h of incubation (Fig. 2A). Cells growing on NB-Glycerol showed initially the lowest fluorescence values, but they increased after 8 hours. In contrast, cells growing with glucose as a carbon source did not show noticeable changes in fluorescence over time, and in general presented very low levels of SigB activation under these conditions (Fig. 2A). To confirm the results obtained from the fluorescence spectroscopy experiment we performed Western blotting using antibodies targeted against the eGFP protein. As expected, the results of the immunoblot matched those of the previous experiment, with NB-Lactose showing the highest level of SigB activation in all time points, NB-Glycerol showing initial low levels of SigB but reaching a peak after 8 h of incubation, and NB-Glucose showing overall low SigB activation levels and no change in expression during the incubation period (Fig 2B).

Fluorescence microscopy of 24-hour cultures grown in NB-Glucose showed very few fluorescent cells, and with low fluorescence intensity. Cultures grown in NB-Glycerol presented a mixed population of non-fluorescent and fluorescent cells. Cells grown in NB-Lactose showed the highest amount of fluorescent cells (Fig. 2C), in line with the results for the quantitative fluorescence spectroscopy and the Western blotting.

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3.3 The impact of carbon sources on stress resistance

To investigate whether the exposure of *L. monocytogenes* to different carbon sources may induce differences in tolerance to lethal stresses, we tested the effect of growth in glucose, glycerol and lactose on the sensitivity of *L. monocytogenes* to lethal acid and heat stresses. Samples were taken at 5 and 24 h of incubation, in order to analyse the stress tolerance of both exponential and stationary phase cells of the three different cultures, since both, actively growing and non-growing cells of *L. monocytogenes* can be present in foods and in food processing environments.

The heat inactivation kinetics of 5-hour grown cells showed that after 20 minutes of 55^{o}C treatment, cells grown in NB-Lactose were significantly more resistant than cells grown in NB-Glucose and NB-Glycerol, with approximately 1 $\log_{10}$CFU/mL higher cell count than the other cultures (Fig. 3A). By the end of the treatment, the cell counts of NB-Lactose were reduced with 2.5 $\log_{10}$CFU/mL reduction, while cells grown in NB-Glucose and NB-Glycerol were reduced to undetectable levels (less than 2 $\log_{10}$CFU/mL) (Fig. 3A). When the same treatment was performed with cells grown for 24 hours, the higher resistance of lactose-grown cells became even more apparent. NB-Lactose cells show no decrease in survival after 30 min of heat inactivation, and the cell counts are significantly higher than those of NB-Glucose and NB-Glycerol cultures showing an approximate 1000-fold higher survival capacity (Fig. 3B). After 45 min of heat inactivation, cells grown in NB-Lactose showed a reduction of approximately 1 $\log_{10}$CFU/mL from the initial cell count while cells grown in NB-Glycerol showed a 4 $\log_{10}$CFU/mL reduction, and cell counts for NB-Glucose cultures were below the limit of detection (Fig. 3B).

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The results of low pH inactivation showed a similar trend as for the heat treatment, although differences between the performance of the cells in the three media were less striking (Fig. 3C and 3D). Cultures incubated for 5 h were treated with a pH of 3, while 24-h cultures showed increased resistance overall, and were therefore treated with a pH of 2 in order to appreciate difference between the conditions. The inactivation curve of 5-h grown cells showed that growth in NB-Lactose induced higher resistance to low pH, with counts of surviving cells between 1.5-2 $\log_{10}$CFU/mL higher than in NB-glucose and NB-glycerol. This difference was significant after 50 and 60 min of treatment (Fig. 3C). In the last sampling point NB-Lactose and NB-Glycerol cells showed a 3 and 4 $\log_{10}$CFU/mL reduction, respectively, while cells grown with glucose showed a reduction in cell counts of 5 $\log_{10}$CFU/mL (Fig. 3C). The 24-h cultures, on the other hand, showed more similar inactivation curves between the different media, although the trend was similar, with NB-Lactose cells being significantly more resistant than NB-Glucose cells after 15 and 60 min of incubation (Fig. 3D).

3.4 The presence of lactose in the media induces higher biofilm formation

It has been previously reported that SigB plays a role in biofilm formation in *L. monocytogenes* (van der Veen and Abee, 2010). Therefore, the impact of the different carbon sources on biofilm formation was tested. The general trend shows that cells growing in NB-Lactose produce more biofilm as measured by CV staining, with the difference between NB-Glucose and NB-Lactose being statistically significant (Fig. 4A). Analysis of biofilm cell counts showed a significantly higher number of culturable cells within the biofilm formed in NB-Lactose, compared to NB-Glucose (more than 100-fold higher) and NB-Glycerol (almost 10-fold higher) (Fig. 4B).

3.5 The impact of different carbon sources on adhesion and invasion of human cells

As SigB has been shown to play a role in virulence (Kazmierczak et al., 2003; Nadon et al., 2002) we performed an infection assay using the human C2Bbe1 cell line with *L. monocytogenes* EGDe cultures grown in NB supplemented with glucose, glycerol or lactose. The percentage of adherent and invasive cells in NB-Lactose (35.3%) was significantly higher than in cells growing in NB-Glycerol (12.6%). Although not significant, cells growing in NB-Glucose also showed a lower percentage of adherent and invasive cells (6.9%) compared to lactose-grown cells (Fig. 4C). In the case of the invasion assay, the differences between the different media were not statistically significant (Fig. 4C).

4. Discussion

In this study, we investigated the effect of three commonly occurring carbon sources, namely glucose, glycerol and lactose, on *Listeria monocytogenes* EGDe growth and activation of the general stress response Sigma factor, SigB, and corresponding phenotypes including stress resistance. Previously Abram et al (Abram et al., 2008) suggested a role for SigB in glycerol metabolism in *L. monocytogenes* strain 10403S whereas we only observed a slightly lower final OD_{600} for the EGDe Δ*sigB* mutant grown in NB-Glycerol. The similarities in growth behaviour of the *L. monocytogenes* EGDe wild-type and its *ΔsigB* mutant strain suggest that SigB does not play a key role in the metabolism of the three tested substrates in these specific conditions possibly due to differences in strain behaviour and the different types of media used. Abram et al used a defined medium with only glycerol as a carbon source (Abram et al., 2008), whereas we used NB as base medium, which likely contains other nutrients as it was shown to allow limited growth of *L. monocytogenes* without carbon

source supplementation (Fig S1). However, we could demonstrate a significant impact of the type of substrate on SigB activation at different growth stages and corresponding phenotypes. The activity of SigB and therefore the activation of the general stress response in *L. monocytogenes,* is regulated by a complex structure known as the stressosome. The stressosome is composed of several proteins that collectively function to integrate environmental stress signals into a pathway that results in the liberation of SigB from an anti-sigma factor, thereby freeing it to participate in transcription of the general stress response regulon. Among the many stresses that SigB helps to protect against are several food preservation-related stresses, including salt, acid, blue light, and nutrient starvation and energy stress (Ferreira et al., 2001; NicAogain and O'Byrne, 2016).

To gain further insight into the relationship between SigB and the metabolism of different carbon sources, several assays monitoring activation of SigB during growth in glucose, glycerol and lactose were performed. Our results showed that SigB activation is lowest during growth in NB-Glucose, and generally higher in NB-Glycerol and highest in NB-Lactose grown cells in both exponential and stationary phase cells. To our knowledge no link between SigB and/or the stress response and the metabolism of the disaccharide lactose has been reported so far in *L. monocytogenes*. According to our growth experiments, the EGDe WT strain and the *ΔsigB* mutant show a similar growth behaviour in NB-Lactose, suggesting that, although the expression of SigB-dependent genes is apparently induced during growth in lactose, the related proteins are unlikely to be essential for lactose metabolism although the exact utilisation pathway is still unclear. As previously described in a study by Pine et al (Pine et al., 1989), *L. monocytogenes* utilises only the glucose moiety for growth, and the galactose moiety is exported outside of the cell via an unknown mechanism. The factors

leading to the activation of SigB in cells actively growing on lactose remain to be elucidated but may be correlated to reduced uptake efficacy and/or metabolism causing energy and/or nutrient stress reflected in the reduced growth performance compared to glucose and glycerol grown cells.

In order to assess the potential of alternative carbon sources to induce resistance to commonly utilised food preservation techniques via SigB-mediated activation of the general stress response in *L. monocytogenes*, several stress treatments were performed. When heat and acid resistance was investigated, a positive correlation between resistance to these stresses and increasing SigB activity was found with cells growing in NB-Lactose consistently more resistant to both heat and low pH than cells growing in NB-Glucose or NB-Glycerol. These results match previously published studies demonstrating a role for SigB in resistance to these stresses (Ferreira et al., 2001; NicAogain and O'Byrne, 2016). Interestingly, a publication by Casadei et al (Casadei et al., 1998) has shown that *L. monocytogenes* (strains ScottA and 1151) is more resistant to heat stress after incubation in dairy products than the same strains growing in TSB broth, although the correlation with SigB activity was not investigated.

It has been shown previously that both biofilm formation and virulence are directly linked with SigB and the stress response in *L. monocytogenes* (Chaturongakul et al., 2008; Kazmierczak et al., 2003; van der Veen and Abee, 2010). It has been suggested that SigB might regulate the expression of some virulence and stress-response genes that are required for survival and multiplication in the host (Chaturongakul et al., 2008; Gahan and Hill, 2014). SigB also regulates transcription of the gene encoding internalin A (InlA) an essential

virulence factor which is crucial for *L. monocytogenes* invasion of epithelial cells. SigB-mediated regulation of InlA has been linked to invasion of Caco-2 cells in vitro and to efficient infection in guinea pigs (Bo Andersen et al., 2007). In the current study the Caco-2/ C2Bbe1 cell line infection assays showed a very clear trend in which lactose-grown cells present higher adhesion and invasion efficacy when compared to cells grown with glucose or glycerol as carbon sources, although in the case of the invasion assay, the differences between the different media were not statistically significant. It seems likely that the increased SigB activity in the presence of lactose causes this increased invasion through an effect on internalin gene expression, but further work will be needed to investigate this.

Additionally, we found an increase in biofilm formation in cells growing in NB-Lactose compared to NB-Glucose and NB-Glycerol, measured by CV (total biofilm production quantification), as well as in culturable cells within the biofilm. The ability to form biofilms is a key factor in the persistence of *L. monocytogenes* in food environments, and an important risk for food safety, as biofilms are a potential source of contamination of food products (Colagiorgi et al., 2017). In general, biofilm cells present higher resistance to environmental stresses, such as antimicrobials or sanitizers, than planktonic cells (Flemming et al., 2016). This suggests that *L. monocytogenes* lactose utilisation capacity may contribute to increased survival in (dairy) food processing environments, resulting in higher recontamination risks and/or growth performance and survival in dairy products. The CV staining assay was performed in polystyrene plates. Although this type of material can be found in food processing environments, it would be interesting to test this phenotype in additional surface materials commonly used in the food industry, such as stainless steel.

To summarise, in this study we identified a potential role for lactose metabolism in the activation of SigB and the stress response in *L. monocytogenes,* conceivably caused by energy and/or nutrient stress-induced triggering of the stressosome. This resulted in an enhanced heat and acid resistance of *L. monocytogenes* in the presence of lactose compared to the other tested carbon sources, as well as increased virulence potential and biofilm production. All these phenotypes triggered by the presence of lactose in the environment may affect stress resistance and infectivity of *L. monocytogenes* along the food chain, although further research is needed to analyse the impact of lactose metabolism during growth in dairy products, as well as the effect of other intrinsic and/or extrinsic factors on this behaviour.

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6. Conflict of interest

The authors declare that they have no conflict of interest.

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8. Legends

Figure 1. Planktonic growth curves. EGDe wild-type (colour) and *ΔsigB* deletion mutant (grey) strains were grown in nutrient broth supplemented with 0.4% of either glucose (A, red circles), glycerol (B, blue squares) or lactose (C, green triangles) at 30°C on a Bioscreen C with continuous shaking. Error bars show the standard deviation of two biologically independent replicates, with three technical replicates each.

Figure 2. Quantification of SigB activity. SigB activity was measured in the WT pKSV7::$P_{lmo2230}$- egfp strain. Cultures were grown on NB supplemented with glucose (red circles), glycerol (blue squares) or lactose (green triangles) at 30^{o}C with 160 rpm shaking. A) Fluorescence intensity corrected for cell density was measured over time using the Spectramax. Error bars show the standard deviation of two biologically independent replicates with three technical replicates each. Values significantly different from NB-Lactose are highlighted with an asterix * ($p \leq 0.05$). B) Protein extracts from cultures grown for 24 h were standardised to 0.8 mg/mL and separated via SDS-PAGE. SigB activity was determined by Western blotting with polyclonal anti-GFP antibodies against the eGFP produced upon activation of SigB. C) 1 mL aliquots of the cultures were pelleted, resuspended in PBS to an OD_{600} of 1 and imaged via phase contrast and fluorescence microscopy (eGFP filter). The scale bar represents 10 µm.

Figure 3. Heat (AB) and acid (CD) resistance. Cultures were grown in NB supplemented with glucose (red circles), glycerol (blue squares) or lactose (green triangles) at 30^{o}C with 160 rpm shaking. Cells were collected after 5 h (AC) and 24 h (BD) of growth and their resistance to (AB) 55^{o}C stress and pH 3.0 (C) or 2.0 (D) was measured over time. Each point represents the

average log_{10}CFU/mL of two biologically independent replicates, and error bars show the standard deviation. The full red circle shows when one of the replicates was below the detection limit (2 log_{10}CFU/mL), and this value was used instead for calculations. When values are missing from the graphs, it means they were below the detection limit. Values significantly different from NB-Lactose are highlighted with an asterisk * ($p \leq 0.05$).

Figure 4. Biofilm formation (A,B) and virulence (C). Cultures were grown on NB supplemented with glucose (red), glycerol (blue) or lactose (green) at 30^{o}C. A) OD_{595} measurements of CV-stained biofilms grown statically on 96-well plates for 48 h. Error bars show the standard deviation of three biologically independent replicates. B) Culturable cells (log_{10}CFU/mL) within biofilms grown statically in 12-well plates for 48 h. Error bars show the standard deviation of two biologically independent replicates. C) The bars show the % of adherent and invading cells in relation to the original inoculum for each medium. Error bars show the standard deviation of two biologically independent replicates. Values significantly different from NB-Lactose are highlighted with an asterisk * ($p \leq 0.05$).

Figure S1. Planktonic growth curves in non-supplemented NB. EGDe wild-type (colour) and *ΔsigB* deletion mutant (grey) strains were grown in plain nutrient broth at 30°C in a Bioscreen C with continuous shaking. Error bars show the standard deviation of two biologically independent replicates, with three technical replicates each.

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Highlights

- ✓ Lactose induces high SigB activity in *L. monocytogenes* EGDe
- ✓ Lactose enhances *L. monocytogenes* EGDe resistance to heat and acid stress
- ✓ Lactose increases biofilm formation and number of culturable biofilm cells
- ✓ Lactose enhances adhesion/invasion capacity of Caco-2-derived C2Bbe1 cell lines

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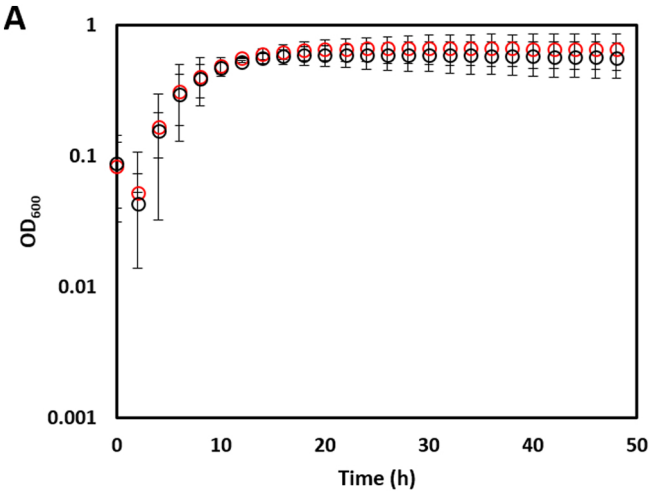
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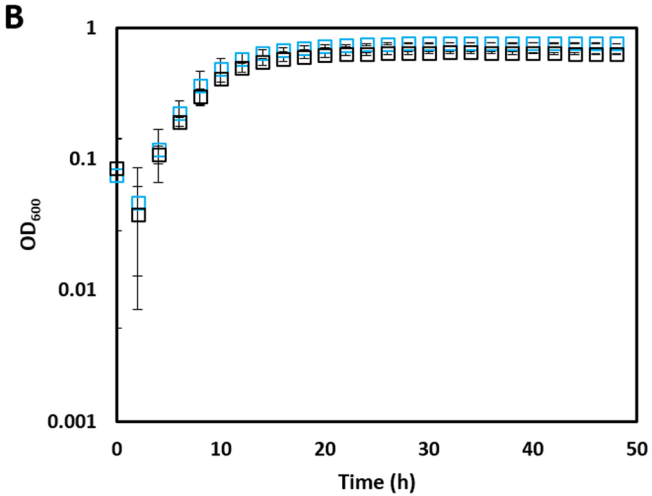
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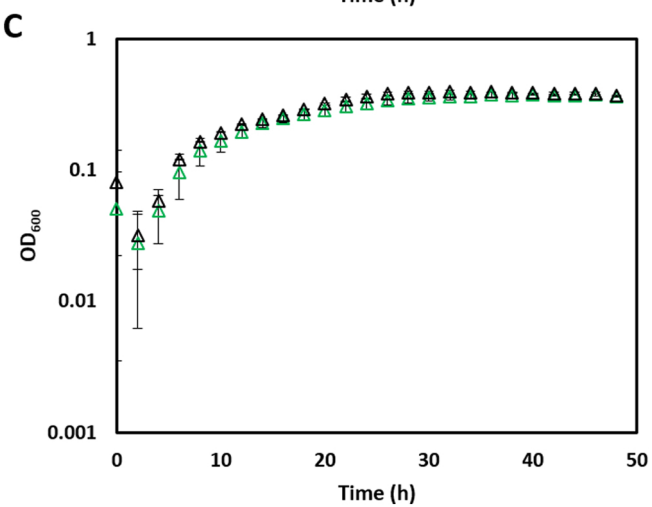
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Figure 1

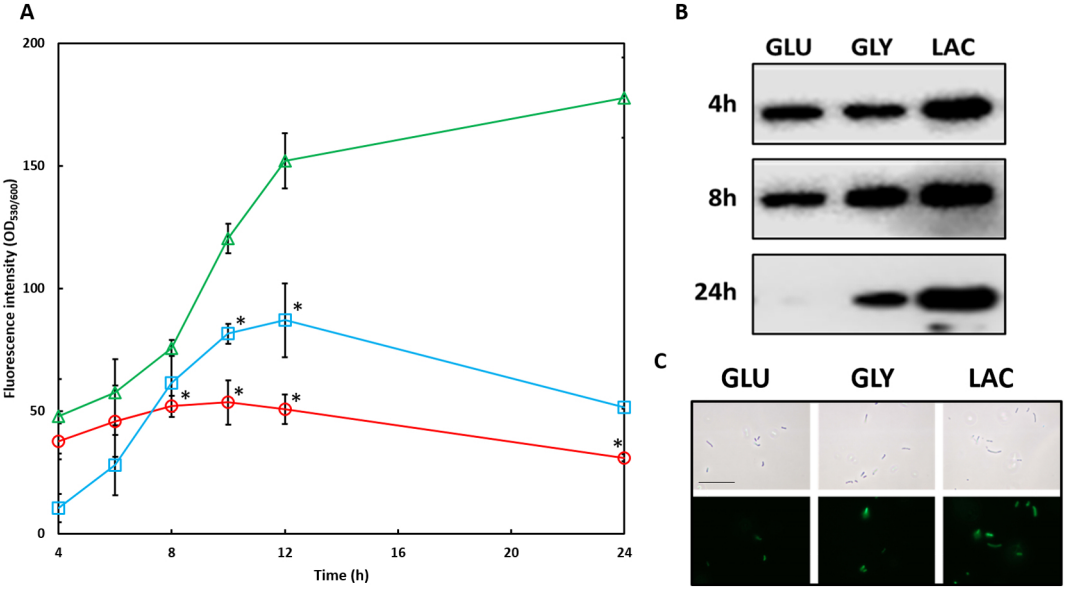
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Figure 2

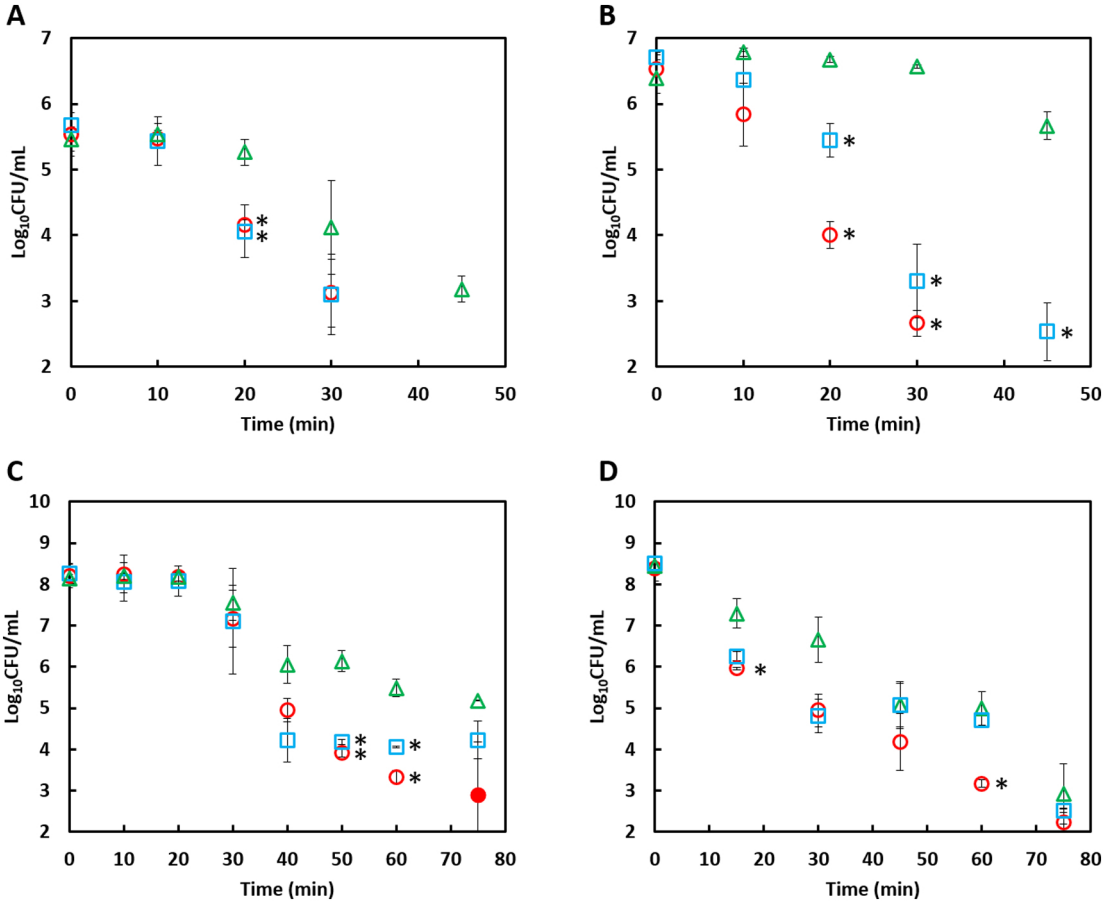

Figure 3

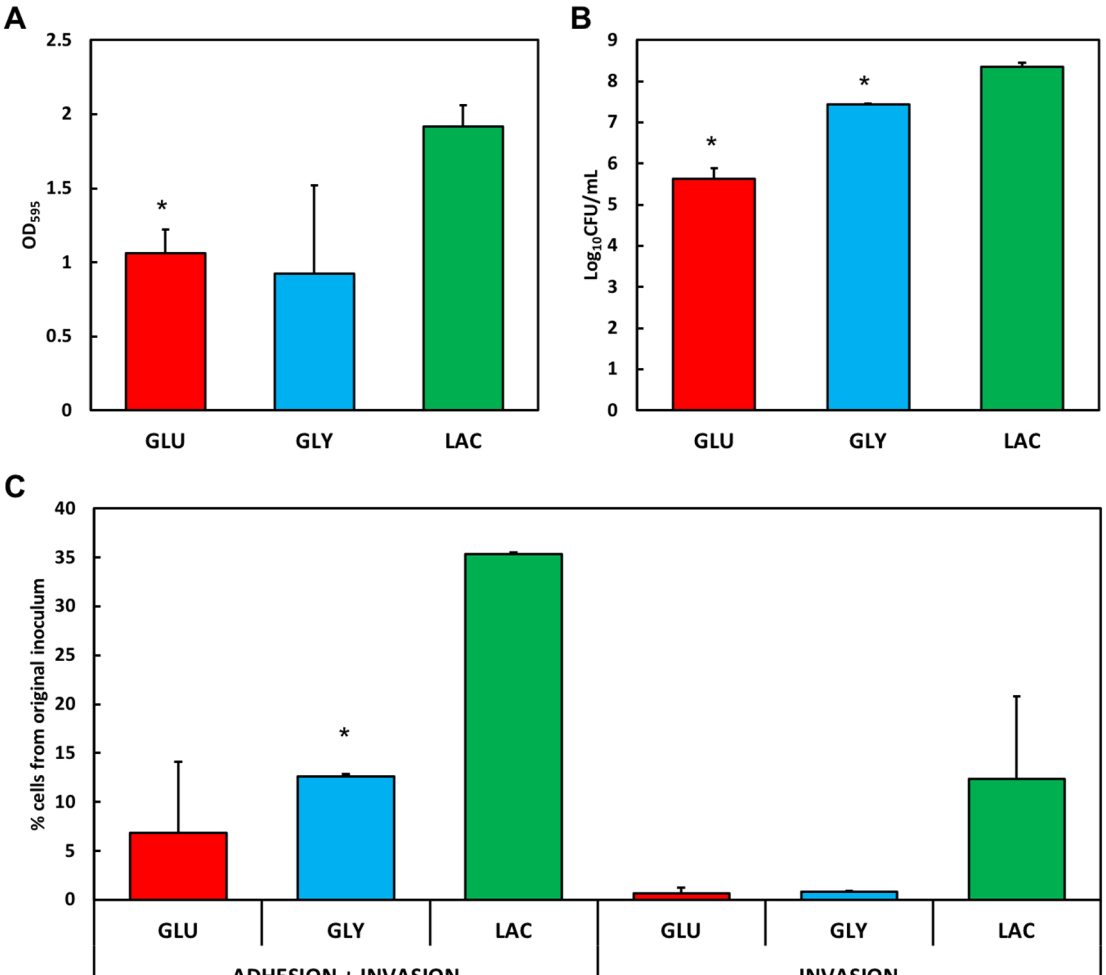
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Figure 4

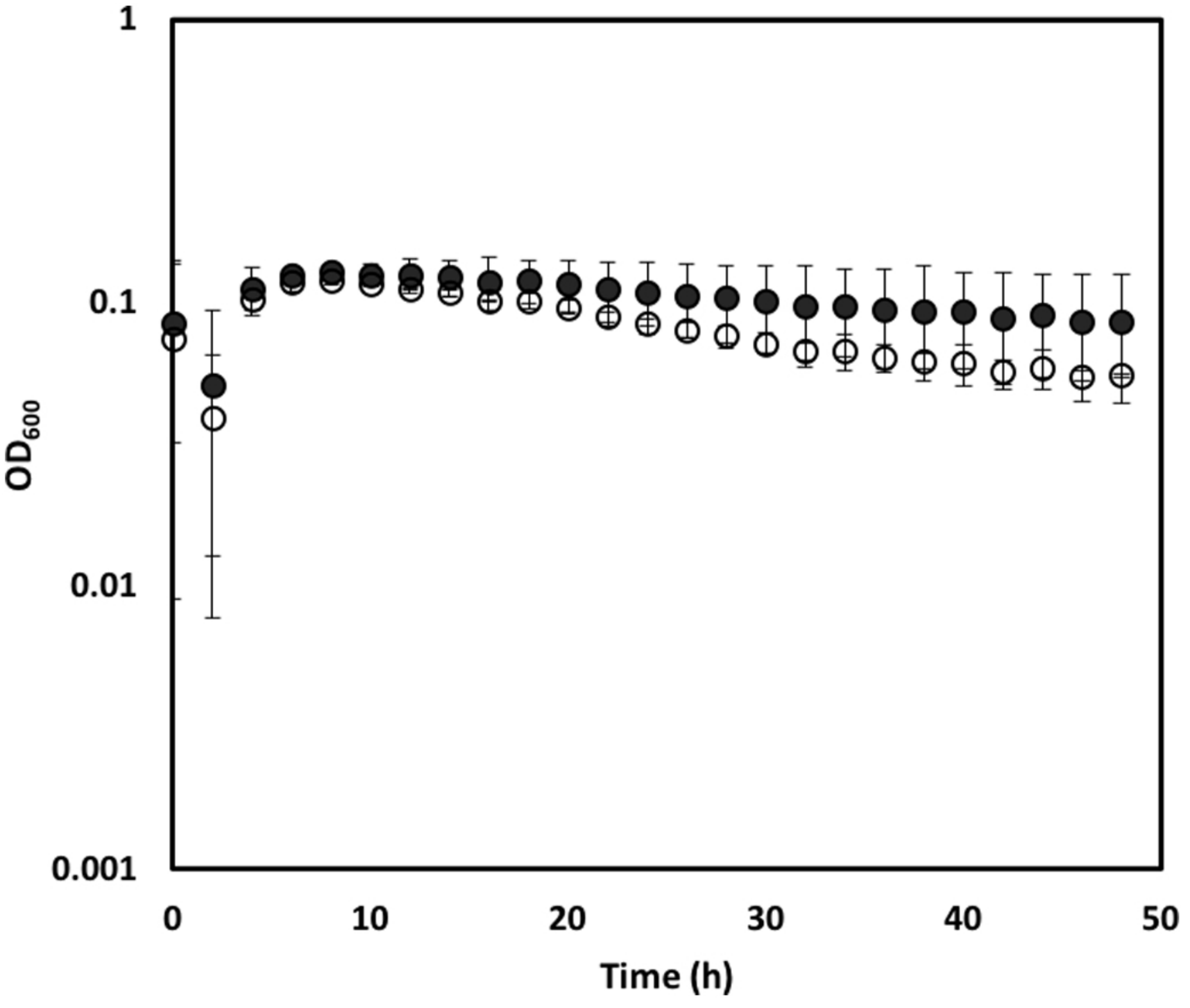

Figure 5