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der Veterinärmedizinischen Universität Wien

Institut für Lebensmittelmikrobiologie
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**Comparison of food-associated *Listeria monocytogenes* growth
phenotypes across sporadic, transient, and persistent strains**

Diplomarbeit

Veterinärmedizinische Universität Wien

vorgelegt von

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Wien, im September 2025

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Wien, den 30.09.2025

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ZUSAMMENFASSUNG

Diese Diplomarbeit konzentriert sich auf die Persistenz von *Listeria (L.) monocytogenes* Stämmen in Lebensmittel produzierenden Betrieben und deren Wachstumsverhalten. Dafür wurden 26 *L. monocytogenes* Stämme aus einem Lebensmittelbetrieb ausgewählt, der verzehrfertige Lebensmittel herstellt, welche besonders anfällig für die Kontamination mit *L. monocytogenes* sind. Dies ist eine ernstzunehmende Problematik, da die Aufnahme und anschließende Infektion mit *L. monocytogenes* zu Listeriose führen kann. Diese Infektionskrankheit kann eine Vielzahl von Gesundheitlichen Problemen verursachen.

Um sicherzustellen, dass die verwendeten Proben tatsächlich *L. monocytogenes* enthalten, wurden alle Proben mit dem selektive Agar RAPID'*L.mono* und der qPCR nach Rossmanith et al. getestet. Eine Probe zeigte widersprüchliche Ergebnisse, mit einem negativen Ergebnis auf dem RAPID'*L.mono* Agar und einem positiven Ergebnis bei der qPCR für *L. monocytogenes*. Daher wurde dieser Stamm mittels *iap* PCR nach Bubert et al., dem selektiv Agar ALOA und der durch Chemilumineszenz gemessenen Enzymaktivität (AquaSpark™) weiter untersucht. Die Ergebnisse zeigten, dass es sich bei diesem Stamm um einen *L. monocytogenes* Stamm mit reduzierter Phospholipase Aktivität handelt.

Für das Hauptexperiment wurden die ausgewählten Stämme in drei Kategorien unterteilt: „persistent“, „nicht-persistent“ und „transient“. Anschließend wurden Wachstumskurven bei Anreicherungstemperatur (37 °C) und Verarbeitungstemperatur (4 °C) im TSB-Y Kulturmedium erstellt. Zunächst wurde versucht die Website dashing-growth-curves.ethz.ch für die Analyse der Wachstumskurven bei beiden Temperaturen zu verwenden. Es stellte sich jedoch heraus, dass sich die Rechenmodell nicht gut an die Wachstumskurven der 4°C Messungen anlegten und deshalb keine verwendbaren Ergebnisse erzielt werden konnten. Daher wurden die 4 °C Wachstumskurven mittels Excel grafisch dargestellt und vergleichend analysiert, während die 37 °C Wachstumskurven mithilfe der Dashing Growth Curves Website und dessen Verwendungen „Gompertz-tight-Modell“ und „Manuell“ analysiert wurden.

Alle Stämme zeigten sowohl unter Anreicherungs- als auch unter Verarbeitungsbedingungen ein gutes Wachstum, aber es konnte unter keiner der beiden Temperaturbedingungen ein relevanter Unterschied im Wachstum zwischen „persistenten“, „nicht-persistenten“ und „transienten“ Stämmen beobachtet werden.

ABSTRACT

This work focuses on the persistence of *Listeria monocytogenes* strains in food production facilities and their growth phenotypes. Therefore, 26 strains of *L. monocytogenes* were selected from a food production facility that manufactures ready-to-eat products, which are prone to contamination with *Listeria*. This is a serious issue, as ingestion and subsequent infection with *L. monocytogenes* can lead to listeriosis, which is an infectious disease that can cause a variety of health problems.

To ensure that the used samples do in fact contain *L. monocytogenes*, all samples were tested using selective RAPID'*L.mono* agar and qPCR according to Rossmanith et al. One sample showed contradictory results, with a negative RAPID'*L.mono* agar and positive qPCR for *L. monocytogenes*. This strain was further investigated using *IAP* PCR according to Bubert et al., selective ALOA agar and the AquaSpark™ chemiluminescence indicating enzyme activity. The results showed that this strain was indeed found to be a *L. monocytogenes* strain with reduced phospholipase enzyme activity.

For the main experiment, the selected strains were divided into three categories: 'persistent', 'non-persistent' and 'transient', and growth curves were created at enrichment temperature (37°C) and processing temperature (4°C) in TSB-Y culture medium. The website dashing-growth-curves.ethz.ch was first used to analyze the growth curves for both temperatures, but no usable results could be obtained for the 4°C growth curves because the calculation models did not fit the growth curve well. Therefore, the 4°C growth curves were analyzed and compared graphically using Excel, while the 37°C growth curves were analyzed using the Gompertz tight model and manually on the Dashing Growth Curves website.

All strains showed good growth under both enrichment and processing conditions, but no major difference in growth between 'persistent', 'non-persistent' and 'transient' strains could be observed under both temperatures.

Acknowledgements

I would like to express my gratitude to all those who have accompanied and supported me on this journey. First and foremost, I would like to thank my supervisors, Dr.rer.nat. Patrick Mikuni-Mester and Lauren Alteio, Ph.D., for their invaluable advice, insightful feedback, and continuous support throughout this research. Their guidance and encouragement were essential to the successful completion of my thesis. I sincerely thank my colleagues at the Institute for Food Microbiology for creating a collaborative atmosphere and for the engaging conversations during lunch. Special thanks go to my lab partner, Victoria Redtenbacher, for her dedication, teamwork, and valuable input during the laboratory work and analysis.

My heartfelt thanks go to my parents for always believing in me and encouraging me to pursue my goals, no matter how long or challenging the journey may have been. Without the love, guidance, and support of my parents and family, completing this degree would not have been possible.

Special thanks go to my friends from university and at home, for their support, encouragement, and for always being there to share both the challenges and the joys of this journey. They made my life at university so much easier and never failed to bring a smile to my face.

Finally, I wish to thank everyone who has contributed, in big or small ways, to making this journey a memorable and meaningful experience.

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Abbreviations

AGES	Austrian Agency for Health and Food Safety GmbH
ALOA	Agar <i>Listeria</i> Ottaviani and Agosti
Aqua dest.	Aqua destillata
BHI	Brain-Heart-Broth
cgMLSTs	core genome Multilocus Sequence Typing types
CI	confidence interval
Ct	Cycle threshold
E	Empty
Fig.	Figure
h	hour/s
HCl	Hydrochloric acid
<i>L.</i>	<i>Listeria</i>
<i>L. monocytogenes</i>	<i>Listeria monocytogenes</i>
MgCl ₂	magnesium chloride
min	Minutes
MLST	Multilocus Sequence Typing
PFGE	Pulsed-Field Gel Electrophoresis
PLC	phospholipase
R	Round
rcf	Relative Centrifugal Force
RLU	Relative Light Unit
RMSE	Root Mean Square Error
RTE	ready-to-eat
S.	Sample
s	seconds
SD.N	Standard deviation
SNP	Single Nucleotide Polymorphism
STs	Sequence Types
TRIS-HCl	Tris-(hydroxymethyl)-aminomethane hydrochloride
TSA	Trypto-Casein Soy Agar

TSB	Trypto-Casein Soy Broth
WGS	Whole Genome Sequencing
Y	Yeast

1 Introduction

1.1. Food safety

Food safety is important for human health, as failure to observe food safety can result in foodborne illnesses (1,2). Human health can be affected by various undesirable food components, such as pathogenic microorganisms (viruses, bacteria, parasites) and their toxins (e.g., cereulide produced by *Bacillus cereus*), chemicals like heavy metals (lead, mercury, cadmium), as well as physical hazards including glass fragments, plastic pieces, or metal objects in processed foods (3–7). The Austrian Food Safety Report 2022 showed that only 0.5% of foodstuffs in Austria were contaminated with contents that are harmful to health (8). Despite this, the EU Zoonoses Report 2023 shows, that foodborne outbreaks still occur regularly in the EU (2). *Salmonella spp.* is the most common cause of foodborne illnesses with 77,486 cases in 2023 and 1,115 foodborne outbreaks (2). The main causes were the consumption of contaminated eggs and egg products, as well as mixed foods and chicken meat and products made from it (2). *Listeria monocytogenes*, on the other hand, is much less common with around 3,000 cases in 2023, but causes more serious illness with hospitalization and possibly even death (2). In order to make food in Austria as safe as possible, food safety monitoring is regulated by the “Food Safety and Consumer Protection Act” (Lebensmittelsicherheits- und Verbraucherschutzgesetz; LMSVG) which is based on EU law, such as the regulation (EC) NO. 2017/625 (9). This regulation defines the principles, requirements, and objectives of official controls along the food chain (9). Responsible for risk analysis, traceability, and rapid alert systems (Rapid Alert System for Food and Feed; RASFF) is the regulation (EC) NO. 178/2002 (10). In particular, the RASFF has the important role of quickly passing on information within the EU to prevent worse consequences in the event of a contamination in the food chain and to reduce risks associated with food consumption (10).

1.2. General information about *Listeria monocytogenes*

Before 2009, it was considered that the *Listeria* genus consisted only of six species, two of which were the considered pathogenic species, *Listeria monocytogenes* and *Listeria ivanovii* while the other four species *Listeria seeligeri*, *Listeria innocua*, *Listeria welshimeri*, and *Listeria grayi* were considered non-pathogenic (11). Between 2010 and 2022, 22 new *Listeria* species were added, but *L. monocytogenes* and *L. ivanovii* remain the only pathogenic species (11,12). *L. monocytogenes* is the most important pathogenic agent, as it is primarily pathogenic to humans, whereas *L. ivanovii* is mainly pathogenic to animals and only rarely human-pathogenic (11).

L. monocytogenes is a gram-positive, rod-shaped bacterium that can survive and grow under adverse conditions, such as high and low temperatures (- 2 to + 45 °C), pH values between 4.2 and 9.5, low moisture content (aw value of 0.92) and high salt concentrations (up to 16 %) in its environment (13). As described above, *L. monocytogenes* is pathogenic and the causative agent of listeriosis in humans and animals and furthermore classified as a zoonosis (see chapter 1.3.1) (2). *L. monocytogenes* is found almost worldwide and occurs in Europe, North and South America, Asia, Africa and Oceania (14). Thereby, the bacterium can be found in many areas of the environment, such as soil and water, but also food and feed (14–16). In particular, the occurrence of *L. monocytogenes* in food production facilities creates a problem, as it can lead to contamination of food and thus pose a health risk to consumers (2,17).

1.2.1. Listeriosis in humans

Human cases of listeriosis have been documented globally, with reports from Africa, Oceania, South America, North America, Europe, and Asia (14). In 2023, there were 2,952 confirmed cases of listeriosis in the EU and 1,497 people were hospitalized and 335 people died (2). Depending on the group of people affected and the course of the disease, the symptoms caused by *Listeria monocytogenes* may vary from flu-like symptoms to symptoms of life-threatening bacteraemia and encephalitis (16). Healthy individuals with a non-impaired immune system usually experience symptoms such as nausea, fever and diarrhoea after consuming food contaminated with *L. monocytogenes* (18). The consumption of contaminated food is in general the most common route of infection and leads to the symptoms mentioned above in healthy adults after an incubation period varying from 6 h to 10 days (16,19). Pregnant

women are particularly at risk of infection, with an approximately 18-fold increased risk compared to the rest of the population (18). This is compounded by the fact that pregnant women often experience severe courses of the disease, including miscarriage, premature labour and stillbirth (20). Even after birth, the newborn can develop serious illnesses such as sepsis and meningitis (20). Another at-risk group are older people over 60 years old and immunocompromised individuals with pre-existing conditions such as cardiovascular disease, chronic kidney disease or lung disease (21). These risk factors, along with some others such as neurological course or previous bacteraemia, lead to an increased mortality rate in cases of *L. monocytogenes* infection (21). To reduce mortality, early diagnosis of the infection and adequate treatment with antibiotics such as penicillin or amoxicillin, often in combination with gentamicin, are important (18).

1.3. *Listeria monocytogenes* in food processing environments

Listeria monocytogenes is currently not only ubiquitous in the environment but is also present in many food processing facilities (2,14). Due to the danger posed by this pathogen as a foodborne zoonosis, there are strict regulations such as Commission Regulation (EC) NO. 2073/2005 (2,22). Fish, meat and sausage processing facilities are particularly affected by contamination with *L. monocytogenes*, but so are facilities that produce cheese from raw milk and facilities that produce fresh, ready-to-eat salads (2). These ready-to-eat food producing companies have the greatest risk of contamination with *L. monocytogenes* as their ready-to-eat products do not need to be cooked or heated before consumption(2). These products are specially protected by the law (EC) No. 2073/2005, which divides these food stuffs into two categories: products in which *L. monocytogenes* can continue to grow and products in which *L. monocytogenes* cannot continue to grow(22). For products in which the pathogen can continue to grow, the manufacturer must prove by means of shelf-life studies that bacterial growth does not exceed 100 CFU/g during the shelf life and *L. monocytogenes* must not be detectable in 25 g of the ready-to-eat product when leaving the premises of the company (22). For foods that do not allow growth, a bacterial count of up to 100 CFU/g is permitted when the product is delivered to the consumer (22). The pathogen can be introduced into the food business both via raw materials such as meat or raw milk, and by staff and inadequate hygiene barriers (23,24). Another source of pathogens is process water and surfaces on which the pathogens can survive for long periods with the formation of biofilms (25,26).

1.4. Persistence in *Listeria monocytogenes*

Persistence of foodborne pathogens in food production facilities is a growing problem for which no definitive solution has yet been found (27). Persistent food pathogens are microorganisms that can survive for long periods of time in food production facilities, despite cleaning and disinfection efforts (13). However, there are currently no genetic markers that can reliably identify a persistent *Listeria monocytogenes* strain as such, and researchers are still searching for a clear definition of the term 'persistence' in the food producing sector (13,27). Studies examining persistence differ both in their methodology and in their definition of persistent strains, which challenges comparison and clear definition (28). Older studies often used Pulsed-Field Gel Electrophoresis (PFGE), which is a method that uses large DNA fragments to produce a characteristic banding pattern ("DNA fingerprint") and thus allows the subtyping and comparison of *L. monocytogenes* strains (29,30). Later, Multilocus Sequence Typing (MLST) analysis was introduced, which is based on 7 housekeeping genes and allows comparison in an open database provided by the Institute Pasteur (29,31). The most recent method is Whole Genome Sequencing (WGS), whereby the data obtained is either analyzed with core genome Multilocus Sequence Typing (cgMLST), whole genome Multilocus Sequence Typing (wgMLST) or single nucleotide polymorphism (SNP) analysis (30,32,33). These newer methods offer the possibility of examining *L. monocytogenes* strains more closely and maybe identify patterns in their genome or genes related to persistence (28). However, these comparative studies require a more precise definition of persistence to detect such patterns (28). While some studies classify strains as 'persistent' and 'non-persistent'. based solely on the frequency of their occurrence in a food production facility, other studies use SNP analysis based on Whole Genome Sequencing (13,28). Unfortunately, there is no uniform definition of the number of matching SNPs required for a strain to be classified as 'persistent' or 'non-persistent' which makes the comparison even more difficult (28). In addition, the samples for analysis are usually obtained from different food businesses and production areas, resulting in highly variable environmental conditions, making studies that do not take them into account difficult to combat (13,17). Furthermore, such environmental factors are important, as they may influence several strategies of *L. monocytogenes* which have been suspected to contribute to its persistence, and these persistence strategies will be addressed in the following section (19,27).

One of the prescience strategies of *L. monocytogenes* is its ability to grow in cold conditions and it makes an ideal pathogen in production facilities, as cold temperatures usually prevail

there (13,34). The bacterium can also survive other negative environmental impacts such as high salt concentrations, low pH values and low humidity (13,35). A frequently mentioned persistence strategy of *L. monocytogenes* is biofilm formation, although the ability to form biofilms alone is not sufficient for effective persistence compared to the growth in mixed biofilms with other species such as *Lactobacillus plantarum* or *Pseudomonas fragi* (13,36,37). For example, the work of Sterniša et al. shows that *Pseudomonas fragi*, a biofilm-forming bacterium, supports the survival of *Campylobacter jejuni* and *L. monocytogenes* at low temperatures of 5-15 °C while the study of Voloshchuk et al. shows that *Pseudomonas spp.* increases the tolerance of *L. monocytogenes* against disinfection (37,38). This is particularly important as biofilms are a common cause of recontamination in food chain and can thus lead to foodborne outbreaks (2,26). The survival of *L. monocytogenes* in protozoa could be a possible persistence strategy, but this strategy has not yet been proven (13,39). What is known, however, is that protozoan species such as *Acanthamoeba* sp. are found in food processing facilities and that *L. monocytogenes* can survive in these protozoan species (39,40). Another persistence strategy discussed is the retarded growth rate, whereby part of the bacterial population slows down its reproduction and metabolism, as bacteria are particularly sensitive to environmental stressors during their exponential growth phase (41). This enables a small part of the population to survive unfavourable conditions and control measures, and these surviving microorganisms reactivate afterwards and start to grow exponentially again (13,42). *L. monocytogenes* and other foodborne pathogen also benefit from the multiple niches in food production facilities that result from areas that are difficult to access and thus challenge adequate cleaning and disinfection (13,27). Drains also represent a particularly dangerous niche, as cleaning can produce aerosols that can contribute to the further spread of *L. monocytogenes* (43). Furthermore, biofilms hide in niches such as cracks, rough surfaces or hard-to-reach areas on equipment and additionally protect pathogens from environmental influences (13,27).

However, despite all the phenotypic advantages attributed to *L. monocytogenes*, no specific trait has yet been identified that characterises certain strains as 'persistent' (13,28).

1.5 Aim of the Study

An important issue in food safety is the persistence of *Listeria monocytogenes* in food producing facilities (19). This raises the question: Is the persistence of *Listeria monocytogenes* in food production related to phenotypic advantages, genetic advantages or extrinsic factors? This study does not claim to answer all parts of this questions but at least covers one important aspect: the phenotypic advantages. Many studies have already investigated the different phenotypic advantages of *L. monocytogenes*, but due to inconsistencies in the origin of the samples, the different investigation methods and the inconsistent definition of the term 'persistence', no comparable results have yet been achieved, and no genetic markers have been found to this date. As described in chapter 1.4, different environmental conditions can lead to inaccurate results and to exclude this sampling bias the samples were taken from only one ready-to-eat food producing company. Subsequently, these 26 selected strains of *L. monocytogenes* were categorised into 'non-persistent', 'transient' and 'persistent' strains by SNP analysis and frequency of occurrences. During this thesis, the strain's growth rates were examined both under enrichment temperature condition of 37°C and at a processing temperature condition of 4°C to achieve comparable results and find phenotypic advantages of certain *L. monocytogenes* strains that could explain their persistence.

2 Material and methods

2.1 Material

2.1.1 Equipment

Tab. 1 Equipment

DESIGNATION	MANUFACTURER
Plate Reader, Tecan Spark	Tecan Group Ltd., Männedorf, Switzerland
Plate Reader, Tecan Infinite 200	Tecan Group Ltd., Männedorf, Switzerland
Pipettes 10µl, 100 µl, 1000 µl	Eppendorf SE, Hamburg, Germany
Safety Bunsen Burner, Schuett phoenix ii	schuett-biotec GmbH, Göttingen Germany
Incubator 37°C, Incucell Eco line	MMM Medcenter Einrichtungen GmbH, Planegg/München, Germany
Vortex Mixer, VWR® VV3	VWR International GmbH, Vienna, Austria
MSC-advantage biological safety cabinet	Thermo Fisher Scientific Inc., Waltham, USA
Centrifuge 5427R	Eppendorf SE, Hamburg, Germany
Thermomixer compact	Eppendorf SE, Hamburg, Germany
Thermocycler, Stratagene Mx3000P	Thermo Fisher Scientific Inc., Waltham, USA
Microwave	Panasonic Industry Austria GmbH, Biedermannsdorf, Austria
GelDoc Go	Bio-Rad Laboratories GmbH, Vienna, Austria
Gel electrophoresis chamber	Analytik Jena GmbH+Co. KG, Jena, Germany

2.1.2 Consumable material + Enrichment and agar media; Chemicals and reagents

Tab. 2 Consumable material

DESIGNATION	MANUFACTURER
Pipette tips 10—20 µl, 100 µl, 1250 µl	AHN Biotechnologie GmbH, Nordhausen, Germany
Nitril gloves	Fisher Scientific, Illkirch-Graffenstaden, France
Disposal bags	Sarstedt AG & Co. KG, Nümbrecht, Germany
Inoculation loops 1 µl	Sarstedt AG & Co. KG, Nümbrecht, Germany
Safe-Lock Tubes 1.5 ml	Eppendorf SE, Hamburg, Germany
Cryogenic vials 2.0 ml	Biologix Europe GmbH, Hallbergmoos, Germany
PCR Tubes	Eppendorf SE, Hamburg, Germany
96-Well Microtiter Plate	Thermo Fisher Scientific, Roskilde, Denmark

Tab. 3 Enrichment and agar media

DESIGNATION	MANUFACTURER
Trypto-Casein Soy Agar (TSA)	Biokar diagnostics, Allonne, France
ALOA Agar	Biokar diagnostics, Allonne, France
RAPID' <i>L.mono</i> Medium	Bio-Rad Laboratories GmbH, Vienna, Austria
Trypto-Casein Soy Broth (TSB)	Biokar diagnostics, Allonne, France
Yeast extract	Biokar diagnostics, Allonne, France
Brain Heart Broth (BHI)	Biokar diagnostics, Allonne, France
Tryptone Soya Broth Plus 0.6% yeast (TSB-Y)	Oxoid Ltd., Basingstoke, England

Tab. 4 Chemicals and reagents

DESIGNATION	MANUFACTURER
Mikrozid AF liquid	Schülke & Mayr GmbH, Norderstedt, Germany
Aqua destillata	Sigma Aldrich, Vienna, Austria
10x PCR buffer	Thermo Fisher Scientific Inc., Waltham, USA
dNTP Mix	Thermo Fisher Scientific Inc., Waltham, USA
PCR Primer	Eurofins Genomics, Ebersberg, Germany
Magnesium chloride (MgCl ₂)	Thermo Fisher Scientific Inc., Waltham, USA
Platinum™ Taq DNA-Polymerase	Thermo Fisher Scientific Inc., Waltham, USA
Chelex® 100 Resin	Bio-Rad Laboratories GmbH, Vienna, Austria
TRIS hydrochloride PUFFERAN® ≥99 %, p.a.	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Agarose	Merck KGaA Darmstadt, Germany
Peq Green	Peglab Biotechnologie Erlangen, Germany
Glycerol	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
AquaSpark™ inositol-phosphate	Biosynth AG, Staad, Switzerland

2.1.3 Bacterial Strains

26 strains were selected prior to this study by my colleague Lauren Alteio, PhD, as can be seen in Table 5. The selection process was as follows: The strains were collected by a ready-to-eat (RTE) food production company over the period from 2018-2023. *Listeria monocytogenes* positive samples were sent to the Austrian Agency for Health and Food Safety GmbH (AGES), where a whole genome sequencing of all samples was performed. In addition, only environmental strains were selected to analyse persistence in the environment. This resulted in a total of 378 strains with 13 sequence types (STs) and 25 core genome MLST types (cgMLSTs)(44). A whole genome single nucleotide polymorphism (SNP) analysis was then performed to further categorize the strains into 'persistent', 'transient' and 'non-persistent' strains. The criteria for the classification 'persistent' were less than ten SNP difference to the other genomes and if the isolate was found at least three times and occurred over a period of at least three years. A strain was declared 'transient' if it showed a difference of 20 or fewer SNP to the other genomes and was found less than three times and over a period of less than three years. The strains that were found one or two times in the entire period were classified as 'non-persistent/sporadic'. To further narrow down the number of isolates for comparison in this study, a representative strain was selected for the different core genome MLST types.

Tab. 5: List of samples with the strain classification, description and ST- And CT-types.

Abbr.	Strain Classification	Name	Year	Description	ST	CT
6	Putatively Persistent	MRL-23-02090	2023	Environmental sample	8	2451
9	Putatively Persistent	MRL-21-00671	2021	Lubricating water/salt bath water	8	3118
17	Putatively Persistent	MRL-23-01259	2023	Environmental sample	8	1358
19	Putatively Persistent	MRL-19-01969	2019	Product-contacting environmental sample	37	2854
22	Putatively Persistent	MRL-23-00876	2023	Environmental sample - Production area	9	2827
23	Putatively Persistent	MRL-23-01298	2023	Environmental sample	8	295
11	Transient	MRL-21-00609	2021	Product-contacting environmental sample	8	5471
15	Transient	MRL-19-00200	2019	Product-contacting environmental sample	204	6692
16	Transient	MRL-22-00249	2022	Product-contacting environmental sample	20	16338
21	Transient	MRL-19-01738	2019	Product-contacting environmental sample	21	2818
24	Transient	MRL-21-01211	2021	Product-contacting environmental sample	37	4252
26	Transient	MRL-23-00636	2023	Environmental sample - Production area	37	16239
1	Non-persistent/ Sporadic	MRL-19-01317	2019	Product-contacting environmental sample	37	8541
2	Non-persistent/ Sporadic	MRL-21-00637	2021	Product-contacting environmental sample	9	92
3	Non-persistent/ Sporadic	MRL-23-00568	2023	Environmental sample - Production area	155	189
4	Non-persistent/ Sporadic	MRL-21-01452	2021	Environmental sample - Wiper	31	15008
5	Non-persistent/ Sporadic	MRL-23-01708	2023	Environmental sample - Production area	37	18973
7	Non-persistent/ Sporadic	MRL-18-02121	2018	Product-contacting environmental sample	6	7650
8	Non-persistent/ Sporadic	MRL-22-00974	2022	Environmental sample - Production area	8	16844
10	Non-persistent/ Sporadic	MRL-23-02591	2023	Environmental sample	77	19422
12	Non-persistent/ Sporadic	MRL-23-00329	2023	Environmental sample - Production area	155	189
13	Non-persistent/ Sporadic	MRL-23-01945	2023	Environmental sample - Production area	14	19038
14	Non-persistent/ Sporadic	MRL-23-00874	2023	Environmental sample	11	18534
18	Non-persistent/ Sporadic	MRL-23-02393	2023	Environmental sample	8	15006
20	Non-persistent/ Sporadic	MRL-22-01152	2022	Environmental sample - Gully	9	15705
25	Non-persistent/ Sporadic	MRL-23-01261	2023	Environmental sample - Production area	1	18724

2.2 Methods

2.2.1 Culturing of *Listeria monocytogenes* strains with RAPID'*L.mono* and TSA Agar

First, the samples were taken from the original culture send by AGES using a 1 µl inoculation loop (Sarstedt AG & Co. KG, Nümbrecht, Germany) and then spread on TSA (Biokar diagnostics, Allonne, France) and RAPID'*L.mono* agar (Bio-Rad Laboratories GmbH, Vienna, Austria). The plates were incubated for 24 h at 37 °C and checked afterwards. Because the colonies on both types of plates were still too small, the plates were incubated for a further 24 h at room temperature. Afterwards, the colonies where big enough for assessment and further processing. For further use, the agar plates were then stored at 4 °C and replaced every four weeks.

Further tests were performed on agar for Sample 4. This Sample was streaked out from overnight cultures as well as from cryocultures on RAPID'*L.mono* (Bio-Rad Laboratories GmbH, Vienna, Austria) and Agar *Listeria* Ottaviani and Agosti (ALOA) (Biokar diagnostics, Allonne, France) and incubated for 48 h at 37 °C. This experiment was repeated on two different days. At the second time the RAPID'*L.mono* (Bio-Rad Laboratories GmbH, Vienna, Austria) plate was split in half and Sample 4 and Sample 6 were spread each on one side (see Fig. 6)

2.2.2 DNA isolation using 2.5 % Chelex® 100 Resin solution

For DNA extraction, a modified protocol according to Walsh et al. was used (45). The Chelex®-TRIS-HCl solution (Bio-Rad Laboratories GmbH, Vienna, Austria; Carl Roth GmbH + Co. KG, Karlsruhe, Germany) was already prepared with 40 ml of 2.5 % Chelex® 100 Resin solution (Bio-Rad Laboratories GmbH, Vienna, Austria) and 10 ml of 0.01 M TRIS-HCl solution (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). 500 µl Chelex®-TRIS-HCl solution (Bio-Rad Laboratories GmbH, Vienna, Austria; Carl Roth GmbH + Co. KG, Karlsruhe, Germany) were pipetted into 1.5 ml Safe-Lock Tubes (Eppendorf SE, Hamburg, Germany) and then one colony from each agar plate to be sampled was taken with an 1 µl inoculation loop (Sarstedt AG & Co. KG, Nümbrecht, Germany) and dissolved in the Chelex®-TRIS-HCl solution (Bio-Rad Laboratories GmbH, Vienna, Austria; Carl Roth GmbH + Co. KG, Karlsruhe, Germany).

Afterwards, the samples were heated at 95 °C (qPCR) or 100 °C (*iap* PCR) for 10 min with the Thermomixer compact (Eppendorf SE, Hamburg, Germany) to lyse the DNA. The samples were then centrifuged with the Centrifuge 5427R (Eppendorf SE, Hamburg, Germany) at 15000 rcf for 30 s for the qPCR and for 10 s for the *iap* PCR. This produced 150 µl supernatant for qPCR and 100 µl supernatant for *iap* PCR, which was transferred to 1.5 ml Safe-Lock Tubes (Eppendorf SE, Hamburg, Germany) for further use.

2.2.3 Quantitative-PCR of all samples

The qPCR was performed according to Rossmanith et al. (46) which targets the *prfA* gene of *Listeria monocytogenes* and even more specific the area between the forward primer LIP1: 5'-GAT ACA GAA ACA TCG GTT GGC-3', the reverse primer LIP2: 5'-GTG TAA TCT TGA TGC CAT CAG G-3' and LIP probe 2: 5'-FAM-CAG GAT TAA AAG TTG ACC GCA-MGB-3'.

For 1200 µl master mix, 492 µl Aqua destillata (Sigma Aldrich, Vienna, Austria) was mixed with 150 µl 10x PCR buffer (Thermo Fisher Scientific Inc., Waltham, USA), 105 µl MgCl₂ (50 mM stock) (Thermo Fisher Scientific Inc., Waltham, USA) 150 µl lip 1FP (5µM stock) (Eurofins Genomics, Ebersberg, Germany), 150 µl lip 2RP (5µM stock) (Eurofins Genomics, Ebersberg, Germany), 75 µl lip-probe-Fam (5µM stock) (Eurofins Genomics, Ebersberg, Germany), 60 µl dNTP Mix (20mM each 5mM stock) (Thermo Fisher Scientific Inc., Waltham, USA) and 18 µl p Platinum™ Taq DNA-Polymerase (5U stock) (Thermo Fisher Scientific Inc., Waltham, USA). Subsequently, 20 µl master mix was mixed with 5 µl of extracted DNA from each sample (see Chapter 2.2.2) and pipetted in two technical replicates. Pure master mix was used as negative control. The samples were then processed with the Thermocycler, Stratagene Mx3000P (Thermo Fisher Scientific Inc., Waltham, USA) using the following program: 94 °C for 2 min, then 45 cycles for 15 s at 94 °C and 1 min at 64 °C. The resulting cycle threshold (Ct) values were then transferred to Excel® (version 2502) from Microsoft® for Microsoft 365 and the mean value for each sample was calculated.

2.2.4 *iap* PCR of Sample 4 (MRL-21-01452)

First the DNA of Sample 4 was extracted as described in Chapter 2.2.2 with a modified protocol according to Walsh et al. (45).

Then the multiplex PCR targeting the *iap* gene was performed according to Bubert et al. (47) with Sample 4 and the primer sequences for each species can be found in this study.

Therefore, the Institute of Food Microbiology at the Veterinary University Vienna provided the laboratory strains of *Listeria spp.* and the master mix already prepared based on the institute's recipe. For the *iap* PCR, the laboratory strains and master mix were thawed and for each *Listeria spp.* 23 µl master mix and 2 µl of the prepared sample were pipetted into PCR tubes (Eppendorf SE, Hamburg, Germany). One with the Sample 4, one with the Chelex® 100 Resin (Bio-Rad Laboratories GmbH, Vienna, Austria) as blank control, four positive controls with *L. welshimeri*, *L. innocua*, *L. monocytogenes*, *L. grayi* and one with Aqua dest. (Sigma Aldrich, Vienna, Austria) as negative control for the PCR. The tubes were put into the Thermocycler, Stratagene Mx3000P (Thermo Fisher Scientific Inc., Waltham, USA) and the *iap* PCR program was started.

Meanwhile, the gel was prepared for gel electrophoresis. Therefore 1.5 g agarose (Merck KGaA, Darmstadt, Germany) was mixed with 100 ml TAE buffer prepared by the Institute of Food Microbiology and heated using the microwave (Panasonic Industry Austria GmbH, Biedermannsdorf, Austria) for 3 minutes. It was waited for that gel to cool slightly before adding 3 µl of Peq Green colorant (Peglab Biotechnologie Erlangen, Germany). Then the gel was poured into the prepared mold and leaved to harden for 30 minutes.

Afterwards the gel was put into the gel electrophoresis chamber (Analytik Jena GmbH+Co. KG, Jena, Germany). Then 5 µl of the marker (100bp) (Thermo Fisher Scientific Inc., Waltham, USA) was pipetted into the outer pocket of the electrophoresis gel. 10 µl of the finished samples were then individually mixed with 3 µl of the marker and pipetted into the single pockets of the gel. Then the 30 min program was started with 120 V, 500 mA and 250 W. Finally, the finished gel was removed, and a photo was taken using the GelDoc Go (Bio-Rad Laboratories GmbH, Vienna, Austria).

2.2.5 Phospholipase enzyme activity of Sample 4 (MRL-21-01452)

To determine the enzyme activity of phospholipase (PLC), in addition to Sample 4, five other samples with different ST types and different growth types (persistent, non-persistent and transient) were selected. The selected samples are Sample 6, 12, 15, 22, 26 and further information on these samples can be found in chapter 2.1.3 in table 5. To create overnight cultures from the six samples, a colony was taken from the prepared TSA plates (Biokar diagnostics, Allonne, France), (see chapter 2.2.1) for each sample and dissolved in TSB-Y (Biokar diagnostics, Allonne, France). These overnight cultures were then incubated for 20 hours at 37 °C. The overnight cultures were then diluted 1:5 with TSB-Y (Biokar diagnostics, Allonne, France) and the OD was measured at 600 nm in a photometer. To achieve an OD of 0.6, these dilutions were then further diluted 1:2 with TSB-Y (Biokar diagnostics, Allonne, France). The enzyme activity in these dilutions was then measured using AquaSpark™ inositol-phosphate (Biosynth AG, Staad, Switzerland) based on the protocol by Bromberger et al. (48). The AquaSpark™ solution (Biosynth AG, Staad, Switzerland) was diluted 1:3 and 1:6 with Aqua dest. (Sigma Aldrich, Vienna, Austria) and then each 40 µl of the AquaSpark™ dilution (Biosynth AG, Staad, Switzerland) and 360 µl of the overnight culture from each Sample were mixed. For the negative control 40 µl of each AquaSpark™ dilution (Biosynth AG, Staad, Switzerland) was mixed with 360 µl TSB-Y (Biokar diagnostics, Allonne, France). Then 150 µl of the sample and the negative control per well in duplicate onto the flat white microtiter plate (Thermo Fisher Scientific, Roskilde, Denmark). After a 10-minute incubation period in the dark, the plate was placed in the Tecan reader Infinite 200 (Tecan Group Ltd., Männedorf, Switzerland) and the following settings were made: Plate flat white without lid, measuring in Kinetic Loop with the mode Luminescence for 46 minutes every 2 minutes. The data was then downloaded and processed using Excel® (version 2502) from Microsoft® for Microsoft 365.

2.2.6 Cryoconservation of *Listeria monocytogenes*

Each sample was cryoconserved as a duplicate, with one part serving as a working culture and one part as a backup culture. For cryoconservation, one colony of each sample was taken from the TSA plates (Biokar diagnostics, Allonne, France) and incubated in TSB-Y (Oxoid Ltd., Basingstoke, England) at 37°C for 18h as overnight culture. The next day, the 2.0 ml Cryogenic vials (Biologix Europe GmbH, Hallbergmoos, Germany) were labelled with name and use (WC for working culture and BC for backup culture). Then 700 µL of the overnight culture and 300 µL glycerol (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) were pipetted into the 2.0 ml Cryogenic vials (Biologix Europe GmbH, Hallbergmoos, Germany) and mixed using the vortex mixer (VWR International GmbH, Vienna, Austria). Afterwards, the cryocultures were frozen and stored at -72°C.

2.2.7 Growth curve

The protocol of M. Aalto-Araneda et. Al. described in “Strain variability of *Listeria monocytogenes* under NaCl stress elucidated by a high-throughput microbial growth data assembly and analysis protocol” (49) was modified and the created protocol was used to prepare the growth curves.

2.2.7.1 Culturing of *Listeria monocytogenes* with TSB culture medium

As previously described in chapter 2.2.1, the 26 TSA agar plates (Biokar diagnostics, Allonne, France) were incubated with the *Listeria monocytogenes* samples and afterwards stored at 4 °C. For the overnight culture a single colony was taken from each plate with an inoculation loop (Sarstedt AG & Co. KG, Nümbrecht, Germany) and then each colony was dissolved in 9ml Tryptone Soya Broth Plus 0.6% yeast (TSB-Y) (Oxoid Ltd., Basingstoke, England). Due to supply difficulties, the TSB-Y culture medium from Oxoid Ltd., Basingstoke, England was switched to TSB and yeast from Biokar diagnostics, Allonne, France during the experiment. The overnight culture was then stored for 18-20h at 37 °C in the Incubator Incucell Eco line (MMM Medcenter Einrichtungen GmbH, Planegg/München, Germany).

For the 37°C growth curves, two biological replicates were incubated for each sample and for the 4°C growth curves three biological replicates were incubated for each sample.

2.2.7.2 Preparation for the growth curve measurement

The next day 10 µl of each overnight culture was diluted into 990 µl of TSB-Y culture medium (Oxoid Ltd., Basingstoke, England; Biokar diagnostics, Allonne, France) to create a 1:100 dilution. Then 10 µl of this dilution was pipetted into each 990 µl of TSB-Y medium (Oxoid Ltd., Basingstoke, England; Biokar diagnostics, Allonne, France) and 990 µl of BHI medium (Biokar diagnostics, Allonne, France) to create a final concentration of 1:10,000. These dilutions were then spread onto 96-Well Microtiter Plates (Thermo Fisher Scientific, Roskilde, Denmark) with 250 µl pipetted into each well. As negative control, 250 µl of each culture medium was then pipetted into four wells to rule out contamination.

For the 37°C growth curves, two biological replicates of each sample were distributed on 96-Well Microtiter Plates (Thermo Fisher Scientific, Roskilde, Denmark) so that they were not next to each other and each replicate was pipetted in duplicate to generate technical replicates of each sample. Therefore, three 96-Well Microtiter Plates (Thermo Fisher Scientific, Roskilde, Denmark) were required for one run to test all 26 samples.

Due to the longer measurement time and the resulting higher risk of contamination, the 4°C growth curves were measured with three technical and three biological replicates each in two experiments. In each experiment, the samples were measured every 24 hours over a period of 480 hours, except on weekends.

2.2.7.3 Growth curve measurement using the Tecan growth curve analyzer

Two different models of plate readers were used due to changing availability. The same program settings were used in both Tecan Spark (Tecan Group Ltd., Männedorf, Switzerland) and Tecan Infinite 200 (Tecan Group Ltd., Männedorf, Switzerland). First the 'Nucleon 96 Flat Transparent microtiter plate' with lid was selected and the area to be measured was specified. For the 37 °C growth curves, the temperature was then set to 37 °C and the option 'Waiting for temperature to be reached' was selected. Then the kinetic loop was chosen with a duration of 20 h, an interval of 20 min and an absorbance wavelength of 600 nm. Before every measurement the plate was shaken for 5 s. This experiment was repeated on two separate days so that each sample was measured at least four times in the two different culture media

TSB-Y (Oxoid Ltd., Basingstoke, England; Biokar diagnostics, Allonne, France) and BHI (Biokar diagnostics, Allonne, France).

Later, five samples were selected and tested again on five following days using the protocol for the 37°C growth curves to exclude time as an influencing factor.

For the 4 °C growth curves, the plates were first measured directly after pipetting. Likewise, the 'Nucleon 96 Flat Transparent microtiter plate' with lid was selected and the area to be measured was specified. Then the absorbance at 600 nm was selected as well as the shaking of the plate for 5 s. This program selection was saved and used for all further measurements. The measurements were taken for approximately 480 h every 24 h except on weekends and between measurements the 96-Well Microtiter Plates (Thermo Fisher Scientific, Roskilde, Denmark) were stored at 4 °C. In the end, each sample was analyzed at least nine times in each of the two different culture media.

2.2.8 Analyzing growth curves with Dashing Growth Curves

The used Software "Dashing growth curves is an open-source web application" which was invented by Michael A. Reiter et. al. and can be found at the following web address <https://dashing-growth-curves.ethz.ch/> (50). The software is used to analyze microbiological growth curves and as an output the user gets the analyzed and visualized data (50).

Therefore, the data needed to be formatted in Excel® (version 2502) from Microsoft® for Microsoft 365 and all data series/measurements required a name (e.g. Sample 1_1) and the negative controls had to be named with blank (e.g. blank 1). In addition, the time had to be specified in hours. An example of the uploaded data can be found in the supplements under Figure S1, which shows the final formatted data for the first measurement of Sample 1 at 37°C. Once the data has been formatted, the Excel file was simply inserted into the website using drag and drop. The data was then automatically analyzed and displayed graphically comparing all samples individually and grouped (see supplemental Figures S2 and S3). For the analysis there were different growth models for selection, such as the Gompertz and Logistic model, as well as the logarithm model Easy Linear (50). The Gompertz and Logistic model could in turn be differentiated into the application Gompertz tight and Gompertz conventional as well as Logistic tight and Logistic conventional (50). In addition, the user could manually select the start and end point of the exponential growth, which could be used if the growth curve shows irregularities that could be misinterpreted by the models (50).

After analysing some of the data with all five models it was decided to use the Gompertz tight model. An example can be found in the supplements as S4. In addition, the growth curves were analyzed manually and therefore a range between 0.1 and 0.4 was selected. For growth curves with lower growth, the range was reduced to a range from 0.1 to 0.3 (see S5). After selection, the website then used the selected model to calculate the growth rate, doubling time, lag time, doublings, doublings in log-phase, yield and the Root Mean Square Error (RMSE). The maximum specific growth rate ($\mu_{\max}$) corresponds to the slope of the steepest section of the logarithmic growth curve and is reached at the tipping point of the curve, while the lag time refers to the period during which the bacteria adapt to the new living conditions (51). Doubling time is the time a bacterial population needs to double its number during the exponential growth phase (52). The number of times a bacterial population has doubled is called “Doublings” and the number of times a bacterial population has doubled during their log-phase is called “Doublings in log-phase” (53). Yield refers to the amount of cellular biomass produced per unit of substrate used during microbial growth and it quantifies the effectiveness with which microorganisms metabolize the available resources (52). Lastly the RMSE quantifies the average deviation between observed and predicted values (54).

The data obtained with the Dashing Growth Curve website was downloaded as csv file and further processed using Excel® (version 2502) from Microsoft® for Microsoft 365. Therefore, the mean value, the standard deviation and the confidence interval were calculated for each sample and each parameter. For a better overview, the mean values were then presented in bar charts and color-coded into three groups: ‘Persistent’, ‘Non-persistent’ and ‘Transient’. Due to larger individual deviations in the analysis using the Gompertz tight model, one result each from Samples 8, 23 and 26 was deleted and the mean value was determined from the remaining measurements.

It was tried to analyze the 4°C growth curves for the 26 samples with Gompertz tight model and manually, but the calculation model did not fit the growth curves optimally. It was therefore decided to evaluate the data graphically using Excel (see Chapter 2.2.7).

2.2.9 Analyzing growth curves with Excel®

The 4°C growth curves were created directly in Excel® (version 2502) from Microsoft® for Microsoft 365. Therefore, the data obtained with the Tecan reader (Tecan Group Ltd., Männedorf, Switzerland) was downloaded as Excel file and for calculation a new Excel file was created. First, the mean value from the blanks of each sample from each of the two measurements was calculated and then subtracted from the measurement data. Next the mean value of each subtracted measurement was calculated and then graphically plotted using a dot plot (see Fig. 6 and 7). Then, the mean value of the standard deviation was calculated for each point and included in the dot plot. To distinguish between the two experiments at the two different timelines, the first experiment is shown with a blue dot plot and the second experiment is shown with an dot plot orange. This resulted in a separate dot plot for each sample, summarised in Fig. 6 and 7.

Three line graphs were then created, each focusing on one of the three categories: 'persistent', 'non-persistent' and 'transient'. To do this, the two mean values obtained from each sample were taken and a further mean value was calculated from them. These mean values were then plotted individually on the graph provided for this purpose and, for comparison, the mean values of all strains in each the other categories were calculated and plotted as a dotted line (see Fig. 8).

3 Results

3.1 Strain confirmation and description

All 26 strains were spread on selective Rapid'L. *mono* agar (Bio-Rad Laboratories GmbH, Vienna, Austria), to confirm the strains as *Listeria monocytogenes*. On this selective agar *L. monocytogenes* shows blue colonies with no halo, while other *Listeria* spp. such as *Listeria ivanovii* show blue colonies with a yellow halo and *Listeria innocua* shows white colonies with no halo (55). Out of the 26 samples that were tested on Rapid'L. *mono* (Bio-Rad Laboratories GmbH, Vienna, Austria) plates, 25 samples showed blue colonies typical for *Listeria monocytogenes* while Sample 4 showed white colonies typical for *Listeria innocua*. After retesting, Sample 4 still showed white colonies on Rapid'L. *mono* agar (Bio-Rad Laboratories GmbH, Vienna, Austria) while another sample (Sample 6) showed blue colonies on the same plate (Fig. 1). Even incubation up to 72 hours at 37 °C and spreading the sample from an overnight culture instead of a cryoculture did not change the result. To see whether Sample 4 also behaved on another selective medium such as *L. innocua*, the sample was spread together with Sample 6 on an ALOA plate (Biokar diagnostics, Allonne, France). As can be seen in Fig. 1, both samples show typical growth for *L. monocytogenes* on ALOA (Biokar diagnostics, Allonne, France), which is a halo surrounding blue colonies, while other *Listeria* spp. do not create a halo (55). Interestingly is the same enzyme phospholipase (PLC), responsible for the blue colonies on Rapid'L. *mono* agar (Bio-Rad Laboratories GmbH, Vienna, Austria), as for the halo around the colonies on the ALOA agar (Biokar diagnostics, Allonne, France)(55). *L. monocytogenes* is the only *Listeria* spp. that produces the enzyme PLC and therefore shows the typical signs on the selective agar (55). This raises the question of whether Sample 4 is perhaps a sample contaminated with *L. innocua* or whether there is something else causing this atypical growth on Rapid'L. *mono* (Bio-Rad Laboratories GmbH, Vienna, Austria).

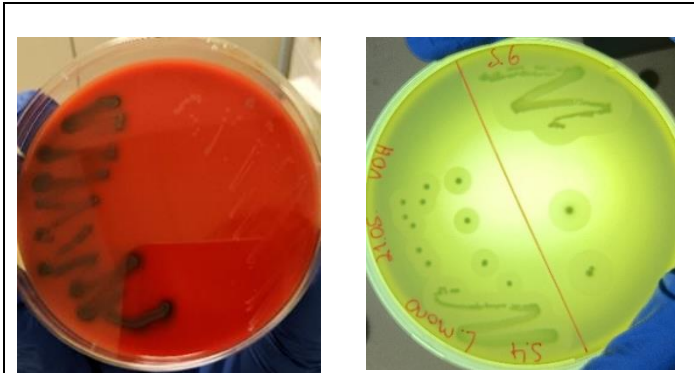


Fig. 1: Rapid'L. mono agar (left side) and ALOA agar (right side) with the Samples 4 (left side of the plate) and Sample 6 (right side of the plate) after incubation.

The qPCR according to Rossmanith et al. was performed to verify whether the samples in fact contain *L. monocytogenes* and whether meaningful experiments can therefore be carried out with these Samples (46). This was particularly important to ascertain after the deviating results of Sample 4 on selective agar. As shown in Tab. 6 the mean CT value of each sample is positive for *L. monocytogenes*, even the mean CT value of Sample 4 is 19.66. The total qPCR shows had a mean CT value of 21.96 and a standard deviation of 1.89. which confirms all samples being *L. monocytogenes*.

Tab. 6: qPCR Mean CT values of each Sample

Sample	1	2	3	4	5	6	7	8	9	10	11	12	13
CTvalue	20.34	21.16	20.54	19.66	20	20.62	24.47	19.2	25.84	21.43	24.08	22.23	19.72

Sample	14	15	16	17	18	19	20	21	22	23	24	25	26
CTvalue	20.39	21.93	22.54	26.93	22.18	21.74	21.84	22.2	23.34	21.09	21.73	24.58	21.08

To compare Sample 4 directly with the *Listeria* spp. (*L. welshimeri*, *L. innocua*, *L. grayi* and *L. monocytogenes*) and to reinforce the confirmation as *L. monocytogenes*, the *iap* PCR was performed according to Bubert et al. (47). The Fig. 2 shows a photo of the gel electrophoresis for the *iap* PCR. As can be seen, Sample 4 (S4) is at the same level as the positive control for *L. monocytogenes*, while the positive control for *L. innocua* and the other *Listeria* species are not at the same level. This confirms that Sample 4 is indeed *L. monocytogenes* and shows that contamination of the Sample was not the reason for the inconclusive results on selective agar. Therefore, a literature search was conducted, and a similar case of white-growing *L. monocytogenes* was found. Based on this, the Sample was further investigated (see below).

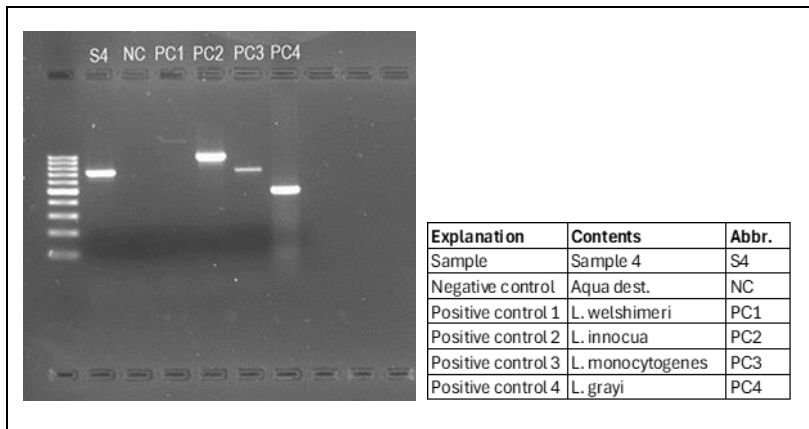


Fig. 2: Foto of the gel electrophoresis from the *iap* PCR for Sample 4.

In this recently published study by M. Stevens et al. describes reduced activity of the enzyme phospholipase as the cause of white-growing *L. monocytogenes* (56). Therefore, it was decided to use the AquaSpark™ inositol-phosphate solution (Biosynth AG, Staad, Switzerland) and the Tecan reader Infinite 200 (Tecan Group Ltd., Männedorf, Switzerland) to measure the relative light units (RLU) according to the protocol from Mester et al (48). These RLU show the activity of the enzyme PLC by chemiluminescence, which is responsible for the blue colour of the *L. monocytogenes* colonies on Rapid'L. *mono* agar (Bio-Rad Laboratories GmbH, Vienna, Austria). As can be seen in Fig. 3 both negative controls (grey dot plots) consisting of AquaSpark™ inositol-phosphate solution (Biosynth AG, Staad, Switzerland) are negative and thus show no contamination. Sample 4 shown in Fig. 3 as red dot plots shows clearly less RLU than the other five strains und therefore less enzyme activity of the enzyme phospholipase. More specifically Sample 4 shows ten to twenty times less enzyme activity than the Samples 6, 12, 15, 22 and even 40 times less enzyme activity than Sample 26. This confirms reduced phospholipase activity as the cause of white-growing *L. monocytogenes* on Rapid'L. *mono* agar (Bio-Rad Laboratories GmbH, Vienna, Austria) in our case as well.

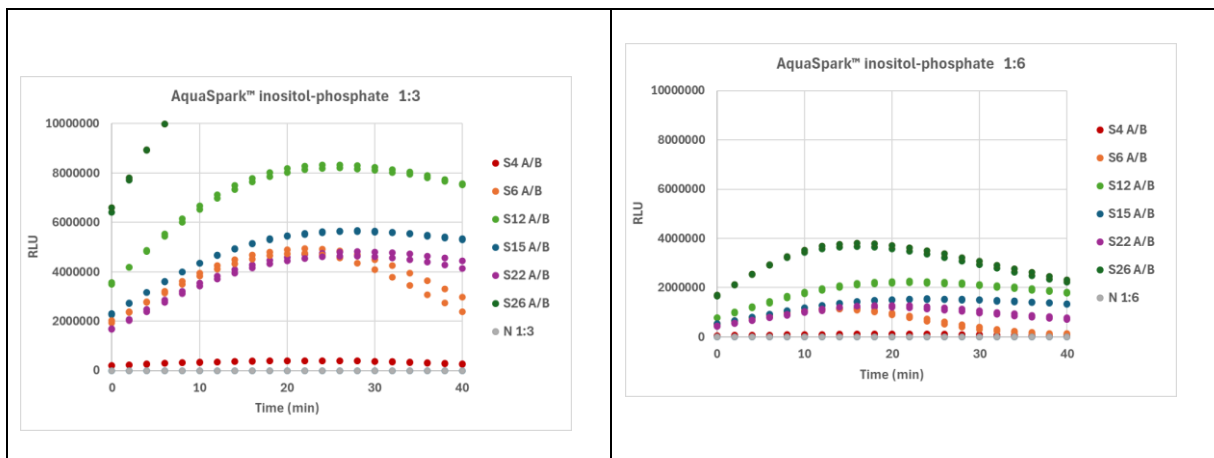


Fig. 3: Measured RLU graphically represented in dot plots as comparison between Sample 4 and the Comparison Samples.

3.2 Growth parameters at 37°C

After all strains were confirmed as *Listeria monocytogenes*, the main growth curve experiment was started. First, the growth curves were determined at enrichment temperature (37°C) in TSB-Y culture medium (Oxoid Ltd., Basingstoke, England; Biokar diagnostics, Allonne, France) and measured with the plate reader Tecan Spark and Tecan Infinite 200 (Tecan Group Ltd., Männedorf, Switzerland). Due to changes in availability, both the TSB-Y culture medium (Oxoid Ltd., Basingstoke, England; Biokar diagnostics, Allonne, France) and the Tecan reader model (Tecan Group Ltd., Männedorf, Switzerland) had to be changed during the experiment. In order to rule out these changes as a source of error, a follow-up experiment was carried out, which will be discussed in more detail in Fig. 5 and the following section. In addition, during the two rounds of the main experiment, two technical and two biological replicates were examined, and four negative controls in the form of TSB-Y culture medium (Oxoid Ltd., Basingstoke, England; Biokar diagnostics, Allonne, France) were measured in each measurement to detect possible contamination. After the measurements, the results were uploaded onto the Dashing Growth Curves website and first analyzed using the Gompertz tight calculation model. However, it became apparent that the calculation model did not optimally fit the growth curves, and it was therefore decided to evaluate the growth curves manually on the Dashing Growth Curve website. The results for the Gompertz tight model can be found in the supplements under S7. The data obtained from the manual analysis using the Dashing Growth Curves website was then downloaded and the mean values of the measurements and replicates were created in Excel® (version 2502) from Microsoft® for Microsoft 365, from which the standard deviation and confidence interval were obtained. This evaluation was carried out for all parameters and it was found that the mean value of the Growth rate ranged between 0.77 h^{-1} and 0.93 h^{-1} with a standard deviation (SD) up to 0.07 and a confidence interval (CI) up to 0.04. The mean Lag time ranged between 7.17 h and 9.71 h with a standard deviation up to 2.0 and a confidence interval up to 1.13. Meanwhile, the mean value was between 0.73 h and 0.91 h based on the Doubling time and the standard deviation was up to 0.08 and the confidence interval up to 0.04. The mean of the Doublings in general ranged from 8.87 to 12.8 while the mean value of the Doublings in log-phase ranged from 1.45 to 1.71. The standard deviation of the Doublings was up to 2.3 while the standard deviation of the Doublings in log-phase was up to 0.22. The Yield mean value varied from 0.53 to 0.85 with a standard deviation up to 0.16 and a confidence interval up to 0.09. The maximum value of the R^2 was 0.999. The exact results of all samples are listed in the table S8 found in the supplements.

For a simpler overview, the results were then presented graphically in the form of bar charts in Excel for the Gompertz tight model (Supplements S9) and manually (Results Fig. 4).

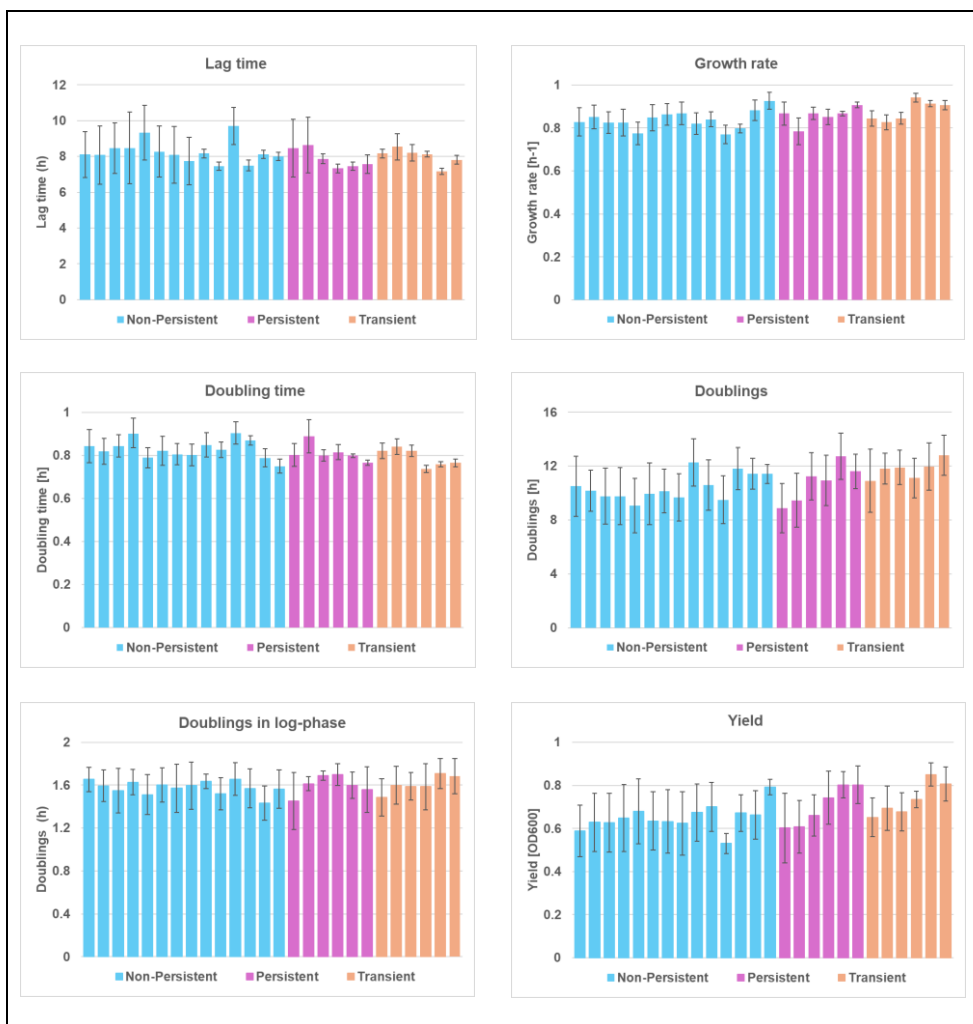


Fig. 4: Bar charts as a graphical comparison of the mean values with standard deviation from each of the 26 strains analyzed manually.

Because the results of the manually selected data had better quality (as described above) compared to the Gompertz tight model data, it was chosen to discuss the manually selected data further in this chapter. The mean values and standard deviation for the manually selected data can be seen in Fig. 4. The persistent strains are shown in purple, the non-persistent strains in blue and the transient strains in orange.

As can be seen in the diagrams, there is no apparent difference between the three categories 'persistent', 'non-persistent' and 'transient' in any of the measured values (Growth rate, Lag time, Doublings, Doublings in log-phase, Doubling time or Yield). Both in the standard

deviations of the bar charts and in the graphical representations of the growth curves on the Dashing growth curve website do the strains show repeatedly large individual deviations that could not be directly explained (see S9 and Fig. 4). To rule out human error, the time interval between measurements and changing equipment as sources of variation, the experiment was repeated with a reduced number of strains (Sample 2, 4, 6, 13, 21) on five consecutive days. Two biological and eight technical replicates were used per day for each Sample and all Samples were cultivated and diluted using the same bottle of TSB-Y (Biokar diagnostics, Allonne, France) and only the Tecan Infinite 200 (Tecan Group Ltd., Männedorf, Switzerland) plate reader. As can be seen in Fig. 5, the strains again showed large biological variance, and no pattern could be identified.

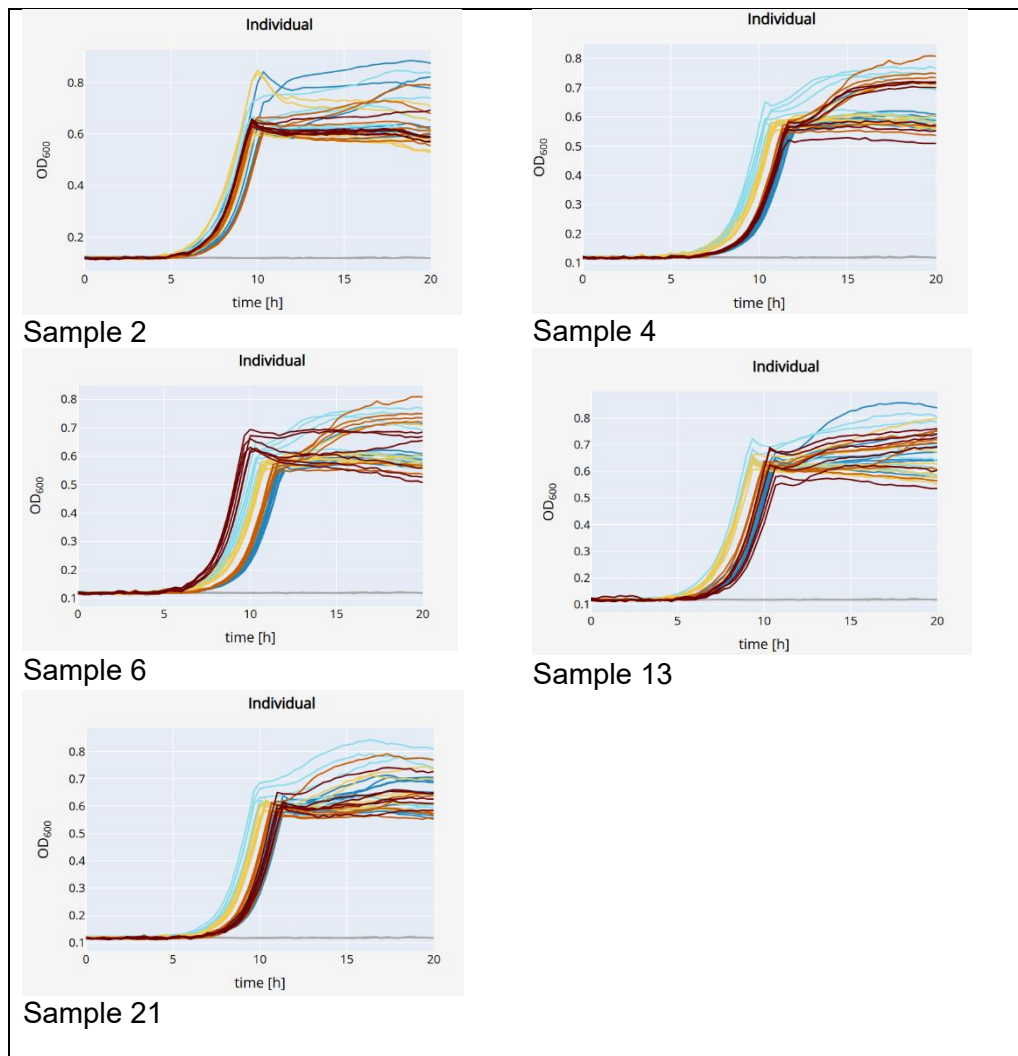


Fig. 5: Screenshots of the Dashing Growth Curve website showing the growth curves in comparison from the five Samples used for the retest.

3.3 Growth parameters at 4°C

To compare growth rates of the 26 strains at processing conditions (4°C), the samples were measurement in two experiments with each three technical and three biological replicates. To simplify the comparison, the mean values of the replicates were calculated from each measurement and these mean values are now shown in Fig. 6 and 7.

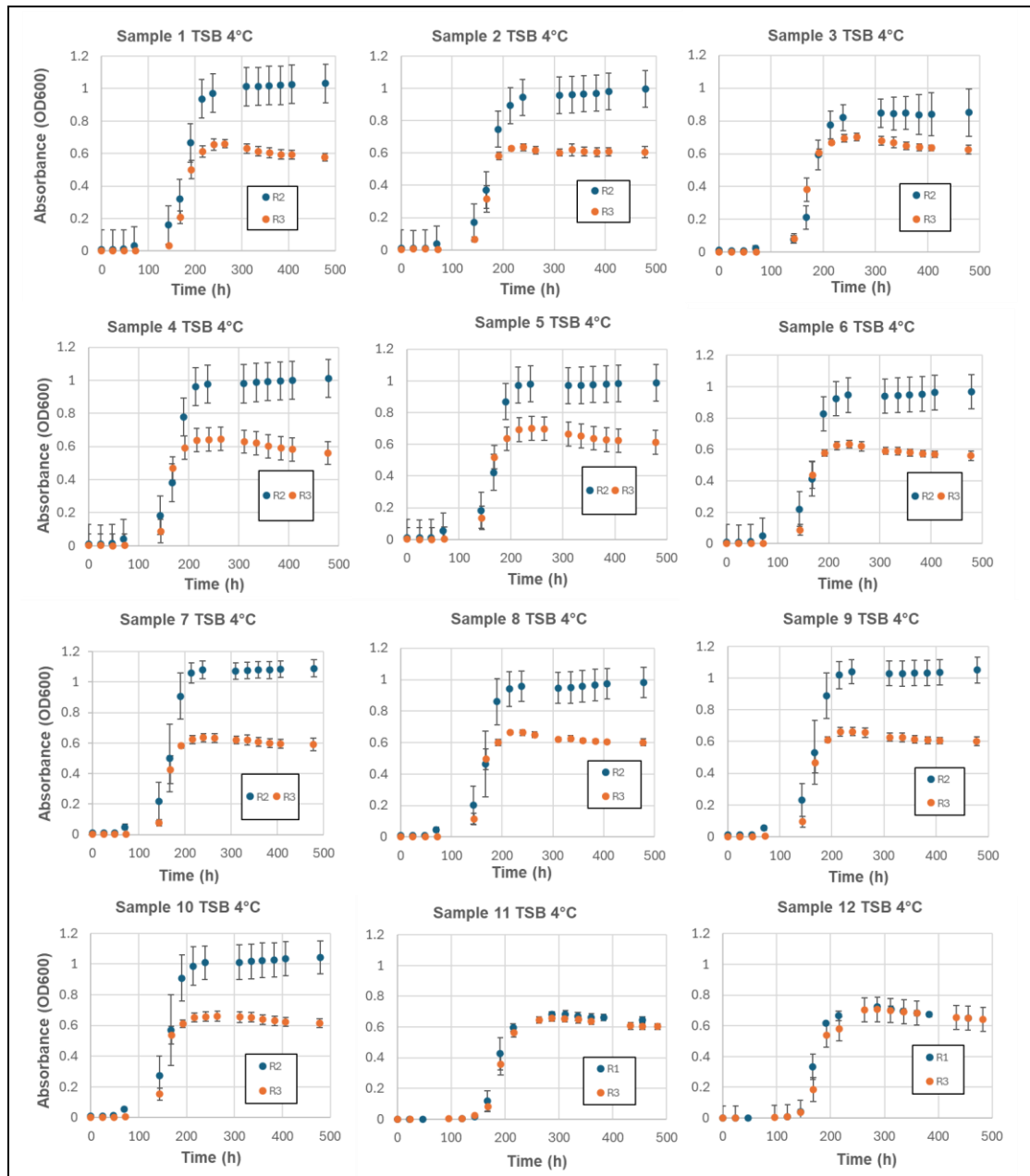


Fig. 6: Results of the 4° C growth curves from Sample 1 – 12 graphically displayed in Excel using dot plot and standard deviation.

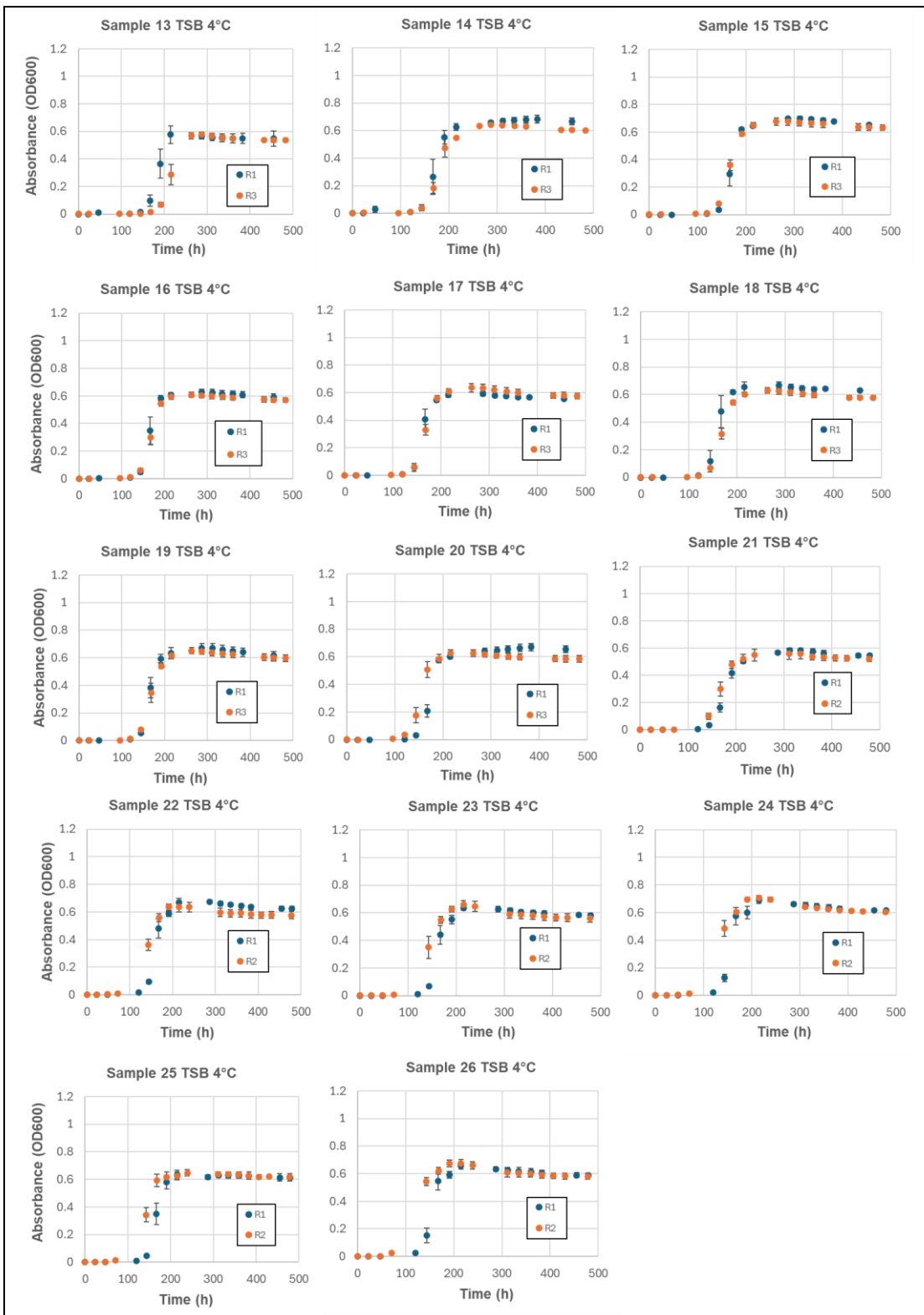


Fig. 7: Results of the 4° C growth curves from Sample 13 – 26 graphically displayed in Excel using dot plot and standard deviation.

First, an attempt was made to analyse the 4 °C growth curves using the Dashing Growth Curves website, as with the 37 °C growth curves, to make both temperatures more comparable. However, after uploading the data to the website, it became apparent that neither the Gompertz tight calculation model nor the manual selection of data could be applied to this data set. The Gompertz tight model did not fit the measured growth curves well, and when selecting the data manually, only a period between two fixed dates could be selected. This meant that all samples showed almost identical results for the different parameters. It is possible that the 24-hour intervals between measurements were too long for this application. For this reason, it was decided to create the 4 °C growth curves shown in Fig. 6 and 7 in Excel® (version 2502) from Microsoft® for Microsoft 365. As described above the mean value of each of the three technical and three biological replicates was calculated in Excel® and then graphically display using dot plots. Each sample was measured over two time periods, which are shown here for comparison as orange and blue dot plots for all 26 strains. The error quotient consisting of the standard deviation is shown as a small black line for each point. There is a certain variance in the two measured periods for strains one to ten, while the remaining 16 strains show graphs that are close to each other. In addition, the first strains show a greater standard deviation than the remaining strains. As can be seen, all 26 strains show exponential growth after 100-150h in both periods despite the cold temperature. Thereby, most strains reach a $\mu_{\max}$ of 0.6 h⁻¹ to 0.7 h⁻¹, while strains one to ten reach a $\mu_{\max}$ of 0.9 h⁻¹ to 1.1 h⁻¹ in their first measurement.

As described at the beginning, the 4 °C growth curves were measured with three technical and three biological replicates over two different time periods, and in addition, four negative controls consisting of the TSB-Y culture medium (Biokar diagnostics, Allonne, France) were measured. These precautions were taken because the samples were measured more frequently and over a longer period of time than the 37 °C growth curves. This increases the risk of contamination and falsification of the results. In fact, contamination of the negative controls was detected during the first measurement of the Samples 1—10 and during the second measurement of Samples 11—20, which is why the results were discarded, and the experiment was repeated.

For better comparison, the strains were divided in Fig. 8 according to one of there three categories, 'persistent,' 'non-persistent,' and 'transient,' and presented as mean values.

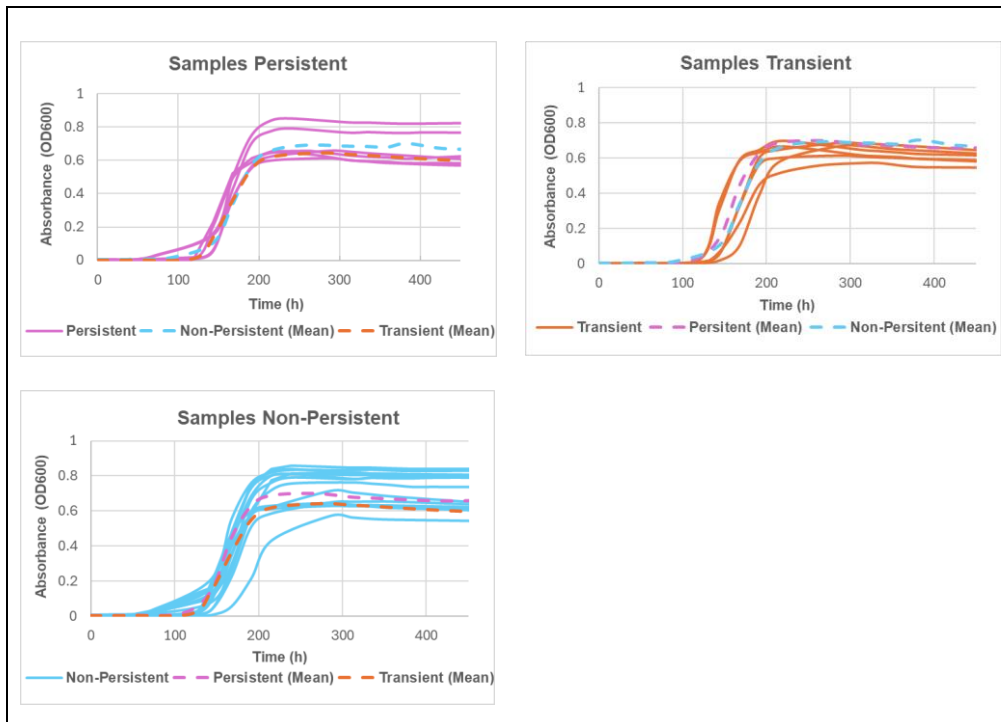


Fig. 8: Growth curves from the mean values of the 26 strains for comparison in 4°C processing conditions.

Figure 8 shows the growth curves of the 26 strains summarized from the mean values of the measurements during the period from 0 to 450 hours. The purple graphs represent the persistent strains, the blue graphs represent the non-persistent strains, and the orange graphs represent the transient strains. The solid graphs represent individual strains, and the dashed graphs represent the mean values of the strains from the two other categories. For example, in the graph at the top left, the purple solid graphs represent the six persistent strains, the dashed blue line represents the mean values from the 14 non-persistent strains, and the dashed orange line represents the mean value from the six transient strains.

from the results it is clear that there is no major difference between the three categories 'persistent,' 'non persistent,' and 'transient.'

4 Discussion

4.1 White *L. monocytogenes* on Rapid'*L. mono* agar

In the course of this thesis, we unexpectedly encountered a strain of *Listeria monocytogenes* that exhibits atypical growth on Rapid'*L. mono* agar (see Chapter 3.1, Fig. 1).

With the results of the qPCR and the *iap* PCR it could be proven, that Sample 4 in fact is *L. monocytogenes* even though it grows with white colonies on Rapid'*L. mono* agar. The reason for this appears to be the considerably lower production of the enzyme PLC which can be seen in Fig. 3. This phenomenon was already described at the beginning of the year by Stevens et al., but they found that the strain on ALOA plates showed a smaller halo which could not be confirmed with strain 4 (56). The discovery of atypically growing *L. monocytogenes* on selective agar is an important finding because Rapid'*L. mono* is regularly used in the testing of *Listeria* samples and can therefore lead to false negative results. Because of the importance of this findings the strains was further investigated, and the results were published in our article "Risk of false-negative diagnostics of *Listeria monocytogenes* due to weak phospholipase activity" (57).

4.2 Comparison of strains at enrichment (37°C) and processing conditions (4°C)

The aim of this study was to investigate the phenotypic differences in growth of the 26 *L. monocytogenes* strains out of one ready-to-eat food producing facility and to discover possible patterns for 'persistence' of certain *L. monocytogenes* strains. It was specifically chosen to only use strains out of one food producing facility because different environments may influence the persistence of *L. monocytogenes* strains and studies often do not take the sampling environment into account (13,17). In addition, a ready-to-eat food business was particularly suitable, as *L. monocytogenes* often persists in these facilities and leads to problems like recontamination and reduced food safety, potentially leading to listeriosis outbreaks (19,25). The strains for this study were divided into three categories: 'persistent', 'non-persistent' and 'transient', and precise definitions were selected for this purpose using SNP analysis and frequency of occurrence to exclude inaccuracies as a source of error. This distinguishes this study from other studies, as different research methods and definitions are often used to investigate the persistence of *L. monocytogenes* which makes these studies

difficult to compare (28). The preparation of growth curves is a reliable method for investigating persistence in *L. monocytogenes*, which is why we also decided to use this method and test the strains at enrichment and processing temperatures (13,17,58). Another important factor was that only environmental samples were selected and no food samples, as these can make the data inaccurate due to re-entry of *Listeria* strains and this does not reflect the state of stationary 'persistence' (17). The strains were adjusted by overnight culture in TSB-Y culture medium (Biokar diagnostics, Allonne, France) to give them all the same starting point for growth to avoid biological variability as an error. Nevertheless, a large biological variability was observed between the strains, which, however, did not reveal any connection between 'persistent' and 'non-persistent' strains (see Fig. 6, 7).

The 2024 published website Dashing Growth Curves was used to analyze the growth curves at enrichment conditions, and the Gompertz tight model and manual selection were therefore selected (50). As discussed previously, the website proved to be not ideal for the analysis of all data sets, but it is a free and easy to use calculation model with many possible applications. This makes it a useful tool for analyzing large data sets of growth curves, but attention should be paid to how the calculation models are fitted to the curves.

The data obtained from the Dashing Growth Curves website were then plotted graphically (see Fig. 4) so that all parameters (Growth rate, Lag time, Doublings, Doublings in log-phase, Doubling time or Yield) could be compared between the three categories. The obtained results revealed no major difference between the 'persistent', 'non-persistent' and 'transient' strains in all measured parameters (see Fig. 4) indicating that bacterial growth rates under optimal conditions do not influence persistence of *L. monocytogenes* in this food production facility.

In order to investigate the growth of the 26 *L. monocytogenes* strains under processing conditions, the strains were incubated at 4°C for approximately 480 hours and measured every 24 hours except on weekends. The resulting growth curves were graphically represented as dot plots using Excel® (version 2502) from Microsoft® for Microsoft 365 and provided with standard deviation (see Fig. 6 and 7), as the attempt to analyze the data using the Dashing Growth Curves website failed, because the calculation model did not fit the growth curves well (see Chapter 3.3). In addition to the graphical representation of each individual strain (see Fig. 6 and 7) the figure 8 was prepared in Excel® using the mean value of each of the three categories 'persistent', 'non-persistent' and 'transient' for better comparison. The graphical representation (see Fig. 6,7 and 8) show no major difference between the three categories,

indicating that persistence of *L. monocytogenes* in this food producing facility is not driven by growth performance under processing conditions.

This is in agreement with the results of the enrichment temperature measurement and shows that the strains do not exhibit any large phenotypic differences in terms of growth. As described above, many different measures were taken to minimise the possibility of errors, which makes this work an important study, even though no phenotypic patterns for 'persistence' of *L. monocytogenes* strains could be found.

5 Conclusion

Coming back to our main question: Is the persistence of *Listeria monocytogenes* in food production related to phenotypic advantages? In terms of growth there was no major difference found between 'persistent', 'non-persistent' and 'transient' strains at both temperatures. Further research is therefore needed to find out whether different phenotypic advantages, genetic advantages or perhaps extrinsic factors lead to persistence in *L. monocytogenes*. Moreover, there is still no clear and uniform definition of 'persistence', which further challenges research and raises the question of whether 'persistence' in the stricter sense exists and can be defined.

The great biological variability between the different strains coming from the same food production facility is another interesting discovery and could be a possible reason for the persistence of *L. monocytogenes*, even though there were no differences between the strains detected.

In addition, it was shown that not all *L. monocytogenes* strains form blue colonies on Rapid'L. *mono*, which is an important point for food safety as it can lead to false negative results. Further research is also important here, both to find the exact cause of this phenomenon and to develop other investigation methods that enable reliable *Listeria* monitoring.

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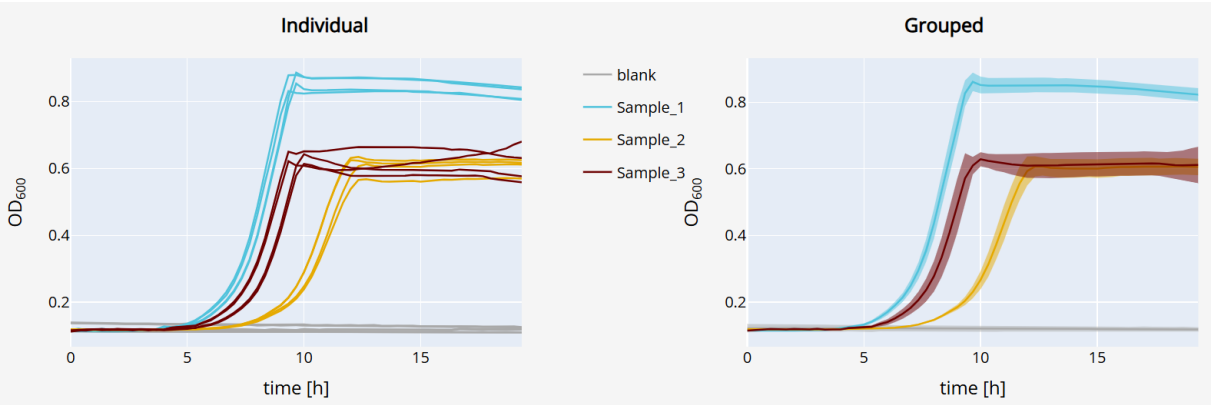
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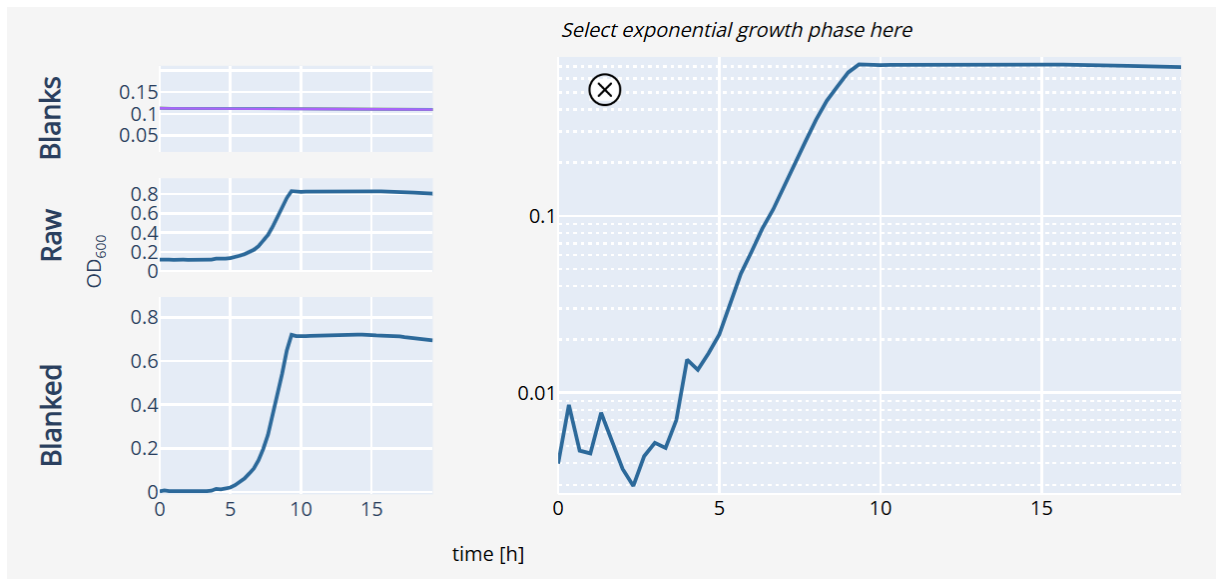
9 Supplements

AN30												
	A	B	C	D	E	F	G	H	I	J	K	L
1	Time [h]	0	0.33333333	0.66666667	1	1.33333333	1.66666667	2	2.33333333	2.66666667	3	3.33333333
2	blank 1	0.1127	0.1123	0.1121	0.1119	0.112	0.112	0.1119	0.1121	0.112	0.112	0.1119
3	blank 2	0.1132	0.1127	0.1127	0.1126	0.1126	0.1125	0.1125	0.1126	0.1125	0.1126	0.1124
4	blank 3	0.1132	0.1127	0.1127	0.1126	0.1126	0.1125	0.1125	0.1126	0.1125	0.1126	0.1124
5	blank 4	0.1371	0.1366	0.13609999	0.13600001	0.13500001	0.13519999	0.1345	0.134	0.1349	0.1338	0.134
6	blank 5	0.1391	0.1391	0.1383	0.13850001	0.13770001	0.13779999	0.1371	0.1364	0.13689999	0.1362	0.1367
7	blank 6	0.138	0.138	0.13689999	0.1372	0.1363	0.1367	0.13609999	0.1349	0.13600001	0.13510001	0.13510001
8	blank 7	0.1126	0.1142	0.1169	0.1179	0.1185	0.1176	0.119	0.1176	0.1173	0.1181	0.1161
9	blank 8	0.1132	0.1154	0.1173	0.1186	0.1192	0.1184	0.1195	0.1191	0.1175	0.1193	0.1173
10	blank 9	0.1147	0.1161	0.1187	0.1191	0.1199	0.1188	0.12	0.1191	0.1186	0.1203	0.1188
11	Sample_1_1	0.117	0.1211	0.1172	0.1169	0.1201	0.1177	0.116	0.1154	0.1167	0.1176	0.1171
12	Sample_1_2	0.1142	0.1137	0.1136	0.1135	0.1137	0.1138	0.114	0.1143	0.1149	0.1156	0.1167
13	Sample_1_3	0.1134	0.1129	0.1128	0.1126	0.1127	0.1128	0.113	0.1133	0.1136	0.1142	0.1149
14	Sample_1_4	0.1145	0.114	0.1138	0.1138	0.1138	0.1139	0.114	0.1143	0.1146	0.1153	0.1161
15	Sample_2_1	0.1185	0.1183	0.1181	0.1181	0.1179	0.1181	0.1179	0.1177	0.1188	0.118	0.1174
16	Sample_2_2	0.1177	0.1176	0.1171	0.1178	0.117	0.118	0.1172	0.1169	0.1178	0.117	0.1174
17	Sample_2_3	0.1177	0.1185	0.1172	0.1173	0.1178	0.1175	0.1172	0.1167	0.1179	0.1182	0.1172
18	Sample_2_4	0.119	0.1193	0.1181	0.1194	0.1188	0.1191	0.1189	0.1189	0.1191	0.1187	0.1191
19	Sample_3_1	0.1156	0.1153	0.115	0.1186	0.1163	0.1166	0.1178	0.1174	0.116	0.1179	0.1155
20	Sample_3_2	0.1124	0.1157	0.1175	0.1178	0.119	0.1173	0.1192	0.1183	0.1175	0.119	0.1176
21	Sample_3_3	0.1145	0.1173	0.1178	0.1189	0.1192	0.1183	0.1199	0.1184	0.1183	0.1202	0.118
22	Sample_3_4	0.115	0.1172	0.1176	0.1195	0.1197	0.1184	0.1196	0.1196	0.1188	0.1209	0.1188

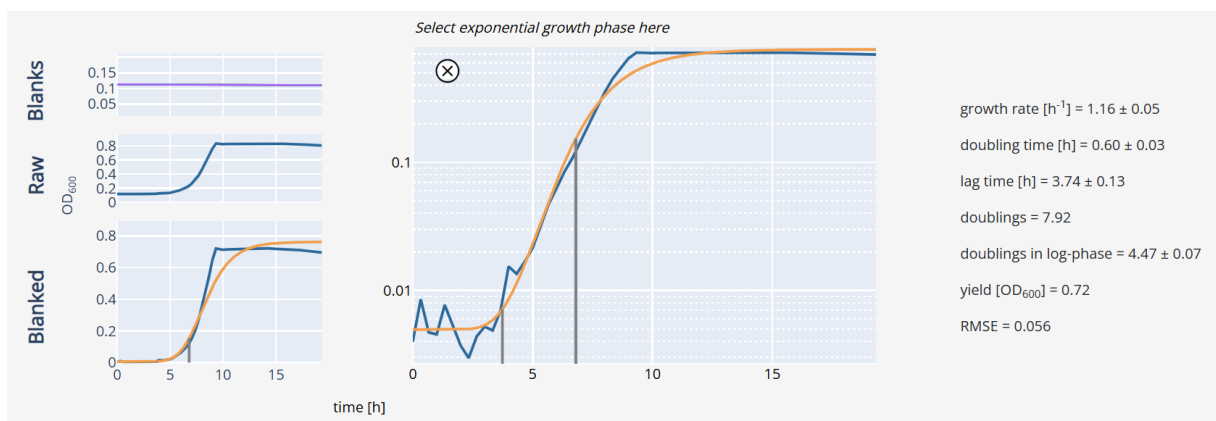
S1: Formatted excel sheet of all three measurements from Sample 1 measured at 37°C.



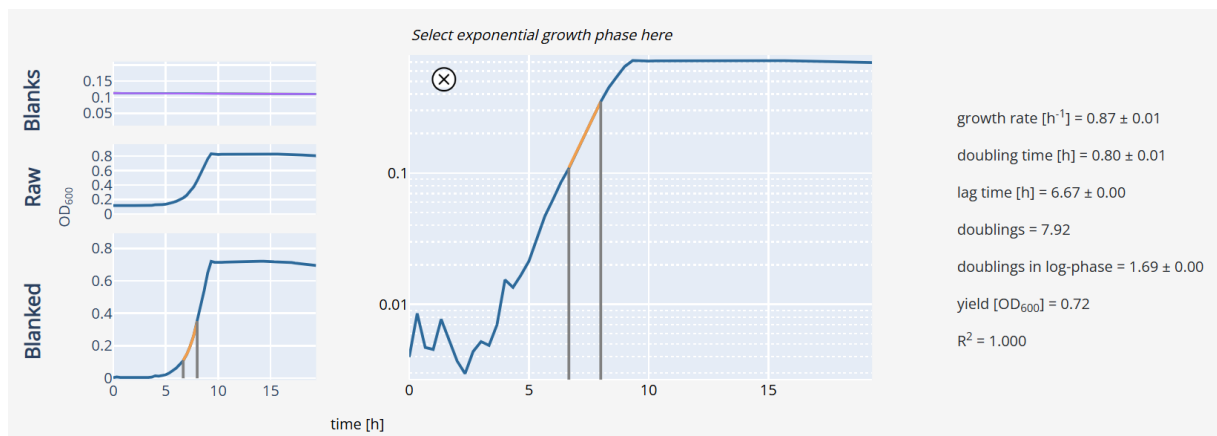
S2: Screenshot of the Dashing Growth Curve website showing all three measurements of Sample 1 measured at 37 °C compared to each other.



S3: Screenshot of the Dashing Growth Curve website showing the first technical and biological replicate of Sample 1 measured at 37 °C named as Sample 1_1.



S4: Screenshot of the Dashing Growth Curve website showing the growth curve from Sample 1_1 analyzed with the Gompertz tight model.



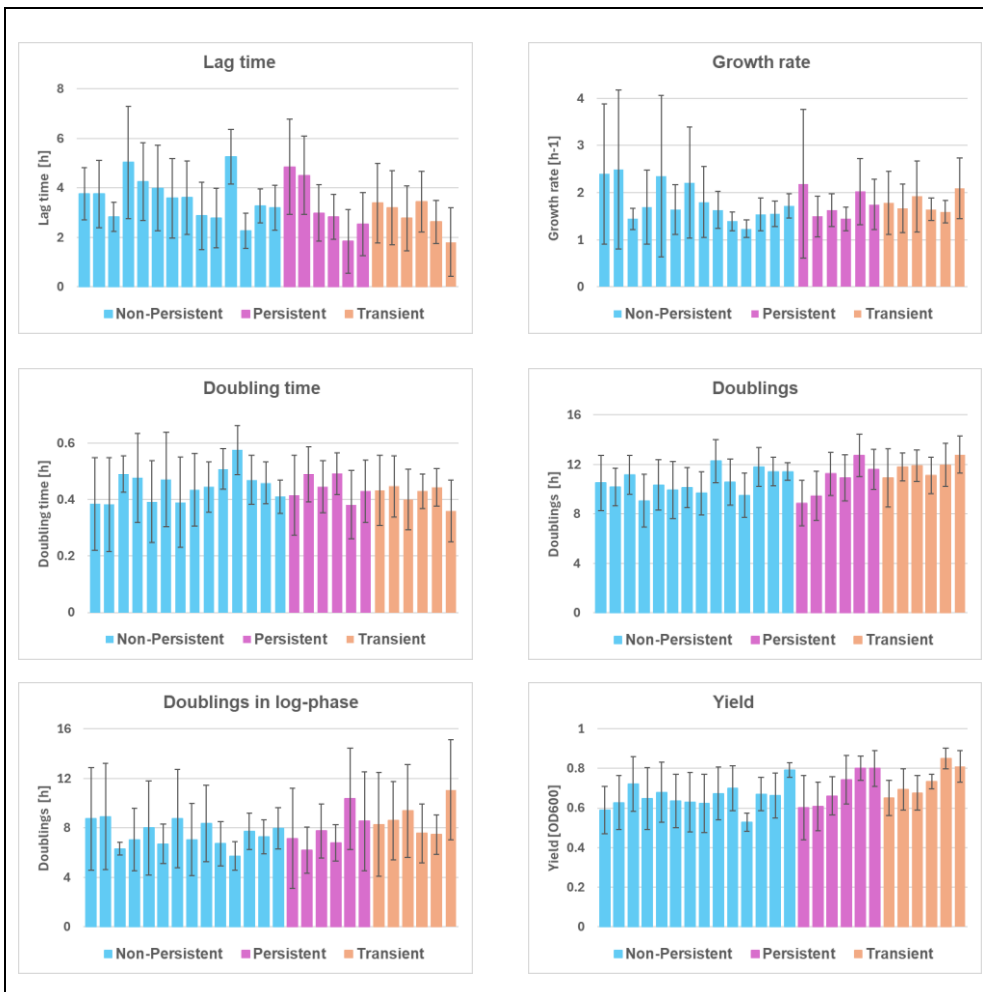
S5: Screenshot of the Dashing Growth Curve website showing the growth curve from Sample 1_1 analyzed with the manually selected tool.

	Growth rate			Doubling time			Lagtime			Doublings			Doublings in log-phase			Yield [OD600]			RMSE		
	Mean	SD/N	CI	Mean	SD/N	CI	Mean	SD/N	CI	Mean	SD/N	CI	Mean	SD/N	CI	Mean	SD/N	CI	Mean	SD/N	
S1	2.395	1.491	0.843	0.385	0.164	0.093	3.762	1.062	2.225	10.503	2.225	1.259	8.736	4.162	2.355	0.589	0.119	0.067	0.062	0.026	
S2	2.49	1.693	0.958	0.383	0.166	0.094	3.752	1.366	0.773	10.181	1.522	0.861	8.923	4.281	2.422	0.628	0.136	0.077	0.072	0.034	
S3	1.442	0.226	0.128	0.491	0.064	0.036	2.838	0.592	0.335	11.157	1.551	0.877	6.317	0.527	0.298	0.722	0.137	0.078	0.083	0.014	
S4	1.689	0.788	0.446	0.477	0.157	0.089	5.034	2.267	1.283	9.072	2.116	1.197	7.045	2.535	1.434	0.648	0.156	0.088	0.058	0.03	
S5	2.344	1.712	0.969	0.393	0.144	0.081	4.256	1.576	0.892	10.338	2.029	1.148	8.001	3.801	2.151	0.68	0.151	0.085	0.073	0.03	
S6	2.184	1.576	0.891	0.415	0.142	0.081	4.851	1.93	1.092	8.872	1.824	1.032	7.141	4.052	2.292	0.602	0.162	0.092	0.063	0.035	
S7	1.641	0.53	0.3	0.472	0.167	0.095	3.99	1.724	0.975	9.944	2.304	1.303	6.711	1.577	0.892	0.635	0.135	0.076	0.066	0.035	
S8	2.21	1.18	0.67	0.39	0.16	0.09	3.58	1.6	0.91	10.14	1.62	0.92	8.75	3.99	2.26	0.63	0.15	0.08	0.07	0.02	
S9	1.497	0.432	0.245	0.49	0.098	0.055	4.508	1.585	0.897	9.463	1.991	1.126	6.202	1.88	1.064	0.608	0.122	0.069	0.067	0.021	
S10	1.798	0.752	0.425	0.435	0.129	0.073	3.614	1.484	0.839	9.675	1.751	0.99	7.059	2.933	1.659	0.624	0.147	0.083	0.063	0.027	
S11	1.784	0.668	0.463	0.433	0.124	0.086	3.383	1.604	1.111	10.911	2.341	1.622	8.281	4.203	2.913	0.651	0.09	0.063	0.074	0.028	
S12	1.633	0.395	0.273	0.445	0.089	0.062	2.87	1.353	0.937	12.278	1.747	1.211	8.349	3.079	2.134	0.674	0.133	0.093	0.081	0.024	
S13	1.39	0.203	0.14	0.509	0.072	0.05	2.776	1.197	0.83	10.585	1.861	1.29	6.733	1.79	1.24	0.7	0.114	0.079	0.07	0.021	
S14	1.234	0.188	0.13	0.576	0.093	0.064	5.256	1.109	0.769	9.506	1.785	1.237	5.734	1.133	0.785	0.53	0.046	0.032	0.054	0.011	
S15	1.668	0.516	0.358	0.447	0.109	0.075	3.195	1.49	1.033	11.81	1.136	0.787	8.584	3.183	2.206	0.694	0.103	0.071	0.076	0.019	
S16	1.92	0.753	0.522	0.401	0.107	0.074	2.77	1.315	0.912	11.898	1.284	0.89	9.375	3.763	2.608	0.677	0.088	0.061	0.071	0.021	
S17	1.625	0.345	0.239	0.446	0.093	0.065	2.989	1.151	0.797	11.232	1.747	1.211	7.748	2.189	1.517	0.661	0.096	0.067	0.082	0.022	
S18	1.538	0.353	0.244	0.47	0.087	0.06	2.263	0.708	0.491	11.812	1.576	1.092	7.742	1.469	1.018	0.671	0.085	0.059	0.081	0.02	
S19	1.445	0.253	0.176	0.492	0.074	0.051	2.839	0.905	0.627	10.928	1.86	1.289	6.8	1.459	1.011	0.742	0.123	0.085	0.082	0.025	
S20	1.552	0.274	0.19	0.459	0.074	0.051	3.267	0.682	0.473	11.427	1.144	0.793	7.279	1.386	0.961	0.663	0.113	0.078	0.081	0.034	
S21	1.647	0.244	0.169	0.43	0.061	0.042	3.435	1.228	0.851	11.111	1.478	1.024	7.552	2.385	1.652	0.734	0.038	0.026	0.096	0.016	
S22	2.024	0.7	0.485	0.382	0.121	0.084	1.84	1.292	0.896	12.738	1.714	1.188	10.352	4.093	2.836	0.802	0.061	0.042	0.096	0.019	
S23	1.75	0.54	0.37	0.43	0.11	0.08	2.53	1.27	0.88	11.6	1.28	0.89	8.54	3.37	2.33	0.8	0.09	0.06	0.09	0.03	
S24	1.595	0.233	0.162	0.444	0.066	0.046	2.622	0.879	0.609	11.966	1.743	1.208	7.464	1.596	1.106	0.85	0.053	0.037	0.101	0.022	
S25	1.722	0.259	0.179	0.411	0.059	0.041	3.203	0.905	0.627	11.422	0.714	0.495	7.971	1.663	1.152	0.792	0.036	0.025	0.109	0.047	
S26	2.09	0.64	0.44	0.36	0.11	0.08	1.81	1.38	0.95	12.8	1.48	1.03	11.08	4.03	2.79	0.81	0.08	0.05	0.1	0.01	

S7: Results at 37° C analyzed with the dashing growth curve website and the selected Gompertz tight model.

	Growth rate			Doubling time			Lag time			Doublings			Doublings in log-phase			Yield [OD600]			R2		
	Mean	SD/N	CI	Mean	SD/N	CI	Mean	SD/N	CI	Mean	SD/N	CI	Mean	SD/N	CI	Mean	SD/N	CI	Mean	SD/N	CI
S1	0.828	0.066	0.037	0.843	0.076	0.043	8.111	1.286	0.728	10.503	2.225	1.259	1.653	0.114	0.064	0.589	0.119	0.067	0.995	0.007	
S2	0.851	0.056	0.032	0.819	0.061	0.035	8.083	1.622	0.918	10.181	1.522	0.861	1.593	0.148	0.084	0.628	0.136	0.077	0.997	0.005	
S3	0.825	0.05	0.028	0.844	0.052	0.029	8.472	1.417	0.802	9.769	2.089	1.182	1.549	0.209	0.118	0.627	0.137	0.078	0.996	0.004	
S4	0.775	0.063	0.036	0.9	0.074	0.042	9.333	2.005	1.134	9.072	2.116	1.197	1.628	0.12	0.068	0.648	0.156	0.088	0.994	0.007	
S5	0.882	0.052	0.029	0.789	0.048	0.027	8.25	1.528	0.865	10.338	2.029	1.148	1.512	0.186	0.105	0.68	0.151	0.085	0.996	0.005	
S6	0.867	0.053	0.03	0.802	0.052	0.03	8.472	1.613	0.912	8.872	1.824	1.032	1.451	0.266	0.15	0.602	0.162	0.092	0.996	0.005	
S7	0.848	0.061	0.034	0.822	0.067	0.038	8.278	1.426	0.807	9.944	2.304	1.303	1.602	0.161	0.091	0.635	0.135	0.076	0.996	0.004	
S8	0.864	0.05	0.028	0.805	0.049	0.028	8.091	1.571	0.889	10.144	1.618	0.915	1.572	0.224	0.127	0.632	0.147	0.083	0.996	0.005	
S9	0.785	0.062	0.035	0.889	0.077	0.044	8.639	1.548	0.876	9.463	1.991	1.126	1.614	0.067	0.038	0.608	0.122	0.069	0.995	0.006	
S10	0.868	0.052	0.029	0.802	0.05	0.028	7.75	1.334	0.755	9.675	1.751	0.99	1.595	0.22	0.124	0.624	0.147	0.083	0.997	0.003	
S11	0.845	0.036	0.025	0.821	0.036	0.025	8.167	0.236	0.163	10.911	2.341	1.622	1.487	0.175	0.121	0.651	0.09	0.063	0.999	0.002	
S12	0.82	0.051	0.035	0.849	0.057	0.039	8.167	0.236	0.163	12.278	1.747	1.211	1.638	0.068	0.047	0.674	0.133	0.093	0.998	0.003	
S13	0.84	0.034	0.024	0.827	0.036	0.025	7.458	0.232	0.161	10.585	1.861	1.29	1.52	0.151	0.105	0.7	0.114	0.079	0.999	0.001	
S14	0.769	0.043	0.03	0.904	0.052	0.036	9.708	1.047	0.725	9.506	1.785	1.237	1.656	0.152	0.105	0.53	0.046	0.032	0.995	0.003	
S15	0.826	0.034	0.024	0.84	0.036	0.025	8.542	0.725	0.503	11.81	1.136	0.787	1.599	0.176	0.122	0.694	0.103	0.071	0.998	0.002	
S16	0.845	0.027	0.019	0.821	0.027	0.018	8.208	0.47	0.325	11.898	1.284	0.89	1.589	0.129	0.089	0.677	0.088	0.061	0.999	0.001	
S17	0.868	0.029	0.02	0.799	0.027	0.019	7.875	0.286	0.198	11.232	1.747	1.211	1.689	0.044	0.03	0.661	0.096	0.067	0.999	0.001	
S18	0.798	0.02	0.014	0.869	0.022	0.015	7.5	0.289	0.2	11.812	1.576	1.092	1.57	0.183	0.127	0.671	0.085	0.059	0.997	0.001	
S19	0.852	0.036	0.025	0.815	0.035	0.024	7.333	0.236	0.163	10.928	1.86	1.289	1.698	0.102	0.071	0.742	0.123	0.085	0.999	0.001	
S20	0.882	0.047	0.032	0.788	0.042	0.029	8.125	0.232	0.161	11.427	1.144	0.793	1.434	0.16	0.111	0.663	0.113	0.078	0.999	0.002	
S21	0.941	0.02	0.014	0.737	0.016	0.011	8.125	0.161	0.112	11.111	1.478	1.024	1.586	0.214	0.148	0.734	0.038	0.026	0.999	0.001	
S22	0.867	0.01	0.007	0.799	0.009	0.006	7.458	0.232	0.161	12.738	1.714	1.188	1.599	0.125	0.087	0.802	0.061	0.042	0.999	0.001	
S23	0.907	0.014	0.01	0.765	0.012	0.008	7.571	0.526	0.364	11.604	1.283	0.889	1.558	0.212	0.147	0.802	0.087	0.06	0.999	0.001	
S24	0.913	0.015	0.01	0.759	0.012	0.008	7.167	0.167	0.115	11.966	1.743	1.208	1.708	0.14	0.097	0.85	0.053	0.037	0.999	0.001	
S25	0.926	0.04	0.028	0.75	0.032	0.022	8	0.236	0.163	11.422	0.714	0.495	1.564	0.18	0.125	0.792	0.036	0.025	0.998	0.002	
S26	0.906	0.021	0.014	0.765	0.018	0.012	7.81	0.243	0.168	12.797	1.481	1.026	1.686	0.164	0.113	0.806	0.078	0.054	0.999	0.001	

S8: Results at 37° C analyzed with the dashed growth curve website and selected manually.



S9: Bar charts as a graphical comparison of the mean values with standard deviation from each of the 26 strains analyzed with the Gompertz tight model.