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Intra- and inter-batch variability in raw pork challenge test studies and their consequences on model predictions:

an intricate interplay between *L. monocytogenes*, the microbiome, and packaging atmosphere

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ABSTRACT

The purpose of this study was to conduct challenge studies in raw pork by strictly following all aspects of the 2014 EURL technical guidance document for conducting shelf-life studies on *Listeria monocytogenes*. Growth potential was assessed on three batches of self-cut pork chops and one batch of in-house prepared pure minced pork without any additives in air and MAP (70% O_2/30% CO_2) packaging. Pork chops did not support the growth of the pathogen throughout the shelf-life, given the specific conditions used in this study, with growth potential values of 0.28 and 0.46 log CFU/g, respectively, for both air and MAP. Substantial growth (>0.5 log CFU/g) was obtained in minced pork after investigating only one batch, with growth potential values of 1.69 and 0.80 log CFU/g, for air and MAP. However, both intra- and inter-batch variability for pork chops and intra-batch variability for minced pork was observed; with elevated growth being evened out by the way growth potential is calculated in the EURL 2014 document, leading to underestimations and posing a potential risk to public health. Maximum growth rate in minced pork at a constant temperature of 7°C was estimated at μ_{max} = 0.680 log CFU/day and μ_{max} = 0.489 log CFU/day in air and MAP, respectively. Model predictions for the growth potential showed acceptable results for air-packed minced pork with better accuracy when the lag phase was implemented as indicated in the renewed protocol (CRL EU, 2021). In MAP, all models used, including the Combase Growth model and to a lesser extent the DMRI dynamic safety model, overestimate the growth potential probably due to a lack of integration of the changing CO_2 levels in the packages. The predictive models used in this study do not adequately account for the dynamics in the raw pig matrix, which may have an inhibitory effect on the growth of *L. monocytogenes*, including interaction with the microbiome and CO_2, and emphasize the importance of remaining critical of predictive model outcomes. In addition, the experimental intra- and inter-batch variability raise questions about the sense or nonsense of using predictive microbiology in these raw pork products.

Keywords: pork chops, minced pork, predictive microbiology, Combase, DMRI

1. Introduction

Pork is globally the second most consumed meat (OECD, 2020) and, consequently, addressing and guaranteeing its safety throughout the agri-food chain up to the consumer is of paramount importance. As with any red meat, pork meat and meat products can be the source of foodborne pathogens, including *Listeria monocytogenes* (Baer et al., 2013). As a result, pork and mainly processed pork products have been implicated in listeriosis outbreaks across Europe in recent decades, primarily affecting individuals with weakened immune systems (Awofisayo-Okuyelu et al., 2016; De Valk et al., 2001; Duranti et al., 2018; Goulet et al., 1998; Pichler et al., 2009). Often, this pathogen is present in raw pork at low or moderate levels (De Cesare et al., 2014; Demaître et al., 2020, 2021), although foods associated with listeriosis outbreaks usually contain *L. monocytogenes* levels above 100 CFU/g (EFSA BIOHAZ Panel et al., 2018; Thévenot et al., 2006). Those increased levels mostly imply the growth of this psychrotrophic pathogen despite the impediments applied during production and conservation processes. Regulation (EC) No 2073/2005 sets a limit for *L. monocytogenes* of 100 CFU/g at the end of shelf life for RTE food products placed on the market during their shelf-life, with a sampling plan of 5 units to be measured during their shelf-life. Hence, this limit also applies to fresh meat consumed raw.

This pathogen has been isolated from pig carcasses with a possible increase in numbers through further cutting and processing (Demaître et al., 2020; Hellström et al., 2010; Nesbakken et al., 1996; Thévenot et al., 2006). The risk attributed to the unavoidable presence along the pork production chain, be it for the pork carcasses, the meat or the environment (Demaître et al., 2021, 2020), emphasizes the importance of appropriate storage and conservation strategies to control or restrict *L. monocytogenes* growth, at processing, retail and the household environment.

Since it has been established that there is a positive association of mainly processed meat products and the risk of foodborne listeriosis (EFSA BIOHAZ Panel et al., 2018; Ross et al., 2009), the prevalence, growth, and risk assessment of this pathogen in various ready-to-eat (RTE) pork products has been assessed in several studies (Dalzini et al., 2014; Hadjicharalambous et al., 2019; Uyttendaele et al., 2009). In contrast, research data on the growth of *L. monocytogenes* on raw pork is scarce, possibly due to the relatively low listeriosis association of these products, as they are, in general, expected to be cooked thoroughly before consumption. On the other hand, raw pork may pose a significant risk if not properly stored, cooked, and handled. The lack of research data is also reflected in the Combase Browser

(available at www.combase.cc): no data could be found on the growth of *L. monocytogenes* with realistic levels of natural microbiota and inoculated pathogens in raw pork cuts and, more surprisingly, also not in minced pork. This latter product is more sensitive, giving its mincing that breaks up cells and leads to leaking of cell content and is classified in Regulation (EC) No 2073/2005 as RTE when consumed raw. Besides, raw minced pork preparations, like preparé, martino, spread, prepared minced meat tartare, are commonly consumed in Belgium. The Belgian food consumption survey of 2014-2015 is the most recent one. That study indicates that about 70% of Belgian men and 60% of Belgian women regularly (meaning: between less than once per week up to once or more per day) eat raw meat preparations including minced pork and minced beef (filet américain) (Lebacq, 2016).

Therefore the aim of this study was to determine (i) the growth potential in pork chops and minced pork under packaging and storage conditions that mirror retail and home conditions, (ii) the maximum growth rate in minced pork at 7 °C, both by strictly following all aspects of the EURL technical guidance document on challenge testing (CRL EU, 2014), the actual one at the moment of testing, and (iii) to make the comparison and interpretation of calculated growth potential and other existing predictive microbiology models versus actual measurements in minced pork. Results will also be discussed in the frame of the new Technical Guidance Document (CRL EU, 2021).

2. Materials and methods

2.1 Strain propagation

The challenge tests were performed using two strains for accounting variations in growth, one reference strain (12MOB085LM, isolated from paté, serogroup IVb) suggested by EURL *Lm* , and one strain from our collection (MB6389, isolated from a beef carcass before chilling in a slaughterhouse, serogroup IIc, CC9; Demaître et al., 2020). Subcultures of the strains were taken from stock cultures at -80°C and cultured separately in Brain Heart Infusion broth (BHI, Oxoid, Basingstoke, England) for 18 h at 37°C to get the cells in the early stationary phase. The strains' purity was checked on Trypton Soy Agar (TSA, Oxoid). Subsequently, starting from the first subculture, a second subculture was incubated at 7°C in BHI broth, of which the concentration was monitored daily to determine the early stationary phase. A temperature profile (T-profile) of 18 h at 37°C followed by 7 days at 7°C was finally determined by performing microbial counts (CRL EU, 2014), which were used to obtain early stationary phase cells in a state that reflects the effect of representing a cold environment (cold-adaptation).

2.2 Growth potential (δ) challenge tests

Three batches were analyzed in the growth potential study (Table 1). The first batch included both chops and minced pork; for the second and third batch, tests were performed on pork chops only. Same day cut pork carrés or loins were purchased from a local producer on the day of the test. Further cutting and mincing were carried out in-house to work standardized. The loin was used to cut pork chops, and the shoulder area of carrés was used to prepare minced pork. No additives were added to the minced pork. For the last two batches, only loins were purchased to cut pork chops.

2.2.1 Preparation test units, inoculation, and storage

Pork chops

Within each batch three loins (replicates) were analyzed, each loin originating from a different carcass. Each loin (about 12 kg) was cut into 13 pork chops of about 2.5 cm thick (± 400 g each); all were placed in separate meat dishes (Nutripack Benelux, model MW1/42, Oss, The Netherlands) and used to prepare the different test units of one replicate: 6 of which were inoculated with *L. monocytogenes*, 6 blanks,

and 1 was used to check natural *L. monocytogenes* contamination. Half of the inoculated and blank test units were air-packed, and the other half-packed under modified atmosphere (MAP). This way, 3 test units, both inoculated and blanks, per replicate and packaging type, were obtained for analysis at day 0, an intermediate day, and at the end of shelf-life. On all 12 test unit pork chops, an area of 25 cm^2 was delineated using a sterile stainless-steel template (5 x 5 cm) and surgical knife (Figure 1).

For the inoculation of each batch a fresh mixed culture of both separately cultivated strains was prepared combined in equal quantity, as indicated in the Technical Guidance Document (CRL EU, 2014). Then the mixed culture was diluted in physiological water to obtain an inoculum solution with an expected concentration of $5x10^4$ CFU/ml. The mixed culture and the pork loins were kept at 7°C. Inoculation was performed at room temperature. The inoculation procedure was identical for all batches, with the delineated surfaces being inoculated with approximately 150 µl of the diluted mixed culture using a micropipette to obtain an inoculum of ca. 300 CFU/cm^2. The volume was evenly spread over the 25cm^2 area using a sterilized drigalski.

Test units were air-packed using cling polyvinylchloride film (Saran, Dow Chemical Company, Zwijndrecht, Belgium) or MAP-packed using a gas packaging machine (VC999 packaging systems Benelux, model TS400, Zonhoven, België). A MAP gas mixture of 70% O_2/30% CO_2 was strived at using a gas mixer connected to the gas packaging machine. High barrier packaging material was used with O_2 permeability of 2 $cm^3/m^2/d$ (OPET/PE-EVOH-PE EPAF, Sarebo, Eindhoven, The Netherlands). The test units followed an industry and consumer-defined storage temperature profile (T-profile), (1) air-packed chops: 2 days at 4°C (manufacturing and retail) and 2 days at 9°C (consumer); (2) MAP chops: 6 days at 4°C (production and retail) and 4 days at 9°C (consumer). The 4°C represents the maximum storage temperature of retail minced meat, and 9°C corresponds to the 75th percentile of Belgian domestic refrigerator storage temperatures (CRL EU, 2014; Devriese et al., 2004). Also, 4°C is the maximum legal retail temperature for minced meat and meat preparations in Belgium, which means that the results of the challenge tests performed are useful for the Belgian retail sector that is supposed to adhere to this temperature.

Pork minced meat

Only one batch was prepared and studied containing three replicates. Per replicate, minced pork was made from the shoulder area of a single carré using a meat grinder (Kenwood, model MG 510, Treviso, Italy). The test units were prepared with 400 (25g/375g or 100g/300g ratios – see further) grams of minced pork placed in meat dishes (Nutripack Benelux, model MW1/42, Oss, The Netherlands) to respect a gas headspace to meat ratio of 2:1. A total of 13 test units were prepared per replicate, of which 6 were inoculated with *L. monocytogenes*, 6 blanks, and 1 was used to check natural *L. monocytogenes* contamination. For the 6 artificially contaminated test units, 25 g of the in-house prepared minced pork to be inoculated and used was set aside from a portion of 375 g of purchased minced pork in each meat dish (Figure 1). For blank test units, the ratio was 100 g of the in-house prepared minced pork and 300 g of purchased minced pork. Only in-house prepared minced pork was used for microbiological analyses, anticipating variations due to subsampling, especially for inoculated samples. The adjacent purchased minced pork in each meat dish only served as a filling to respect the gas headspace and thus mimic the commercial food product proportions meat/gas as closely as possible. Under no circumstances does the purchased minced meat come into contact with the portions for analysis. Similar to the pork chops, half of the inoculated and blank test units were air-packed, and the other half MAP, resulting in 3 test units, both inoculated and blanks, per replicate and per packaging type for analysis at day 0, an intermediate day, and at the end of shelf-life.

The same mixed culture obtained and diluted for pork chops was used for minced pork inoculation. The in-house prepared minced pork (25 g) was inoculated in-depth with the diluted, mixed culture (5×10^4 CFU/ml) using a micropipette to obtain a concentration of 2500 CFU/25 g (100 CFU/g). The diluted, mixed culture (50 µl) was inoculated dropwise to multiple sites, after which the minced pork was mixed using the micropipette tip to disperse the inoculum over the outside and inside of the 25 g. Product packaging was carried out in the same way as described for pork chops using the same materials and gas composition.

Test units followed an industry-defined T-profile, (1) air-packed mixed meat: 2 days at 4°C (manufacturing and retail) and 2 days at 9°C (consumer); (2) MAP mixed meat: 5 days at 4°C (production and retail) and 2 days at 9°C (consumer).

2.2.2 Microbiological analyses

On day 0, on the intermediate time point when the temperature in the profile was changed and at the end of the shelf-life, microbiological analyses were performed. At each test moment, *L. monocytogenes* numbers were determined on the inoculated test units, while the microbiome and physicochemical characteristics (a_w, pH) were determined for the blank test units.

For each pork chop, the 25 cm^2 delineated area was analysed by cutting out the total surface (approximately 0.4 cm thick) of the area with a sterile surgical knife and forcep, resulting in a meat sample of 10 g. Each pork chop subsample was placed in a stomacher bag, and 90 ml Buffered Peptone Water (BPW, Oxoid) was added to quantify *L. monocytogenes* in the inoculated test units or the microbiome in the blank test units. Additionally, measurements for a_w and pH were performed on the surface next to the cut out area of the blank pork chops.

For minced pork, the total 25 g inoculated test samples were analyzed to enumerate *L. monocytogenes*. From the blank test units, a 25 g sample was collected to determine the microbiome, leaving the remaining 75 g for a_w and pH measurements. Each 25 g minced pork sample was supplemented with 225 ml Buffered Peptone Water (BPW, Oxoid) in a stomacher bag and homogenized for 2 min using a Stomacher Lab Blender 400 (Seward Laboratory, London, United Kingdom). Enumeration of *L. monocytogenes* in the inoculated samples was performed according to ISO 11290-2.

Absence/presence of *L. monocytogenes* in 25 g was also tested at day 0 using ISO 11290-1.

Total aerobic psychrotrophic bacterial counts (TAC) were obtained using plate count agar plates (PCA; Oxoid) incubated at 22 °C for 5 days. Psychrotrophic lactic acid bacteria (LAB) were determined using Man Rogosa Sharp Agar plates (MRS; Oxoid) incubated at 22°C for 5 days. Presumptive *Pseudomonas* spp. were enumerated on *Pseudomonas* agar plates (agar base Oxoid, CFC selective agar supplement Oxoid) incubated at 30°C for 3 days. Oxytetracycline glucose yeast extract agar incubated at 25°C for 5 days was used for the enumeration of yeasts and molds (Y&M) (OGYE; agar base Oxoid, oxytetracycline GYE selective supplement Oxoid).

2.2.3 Intrinsic and extrinsic parameters

On each test moment, the a_w and pH were determined. pH measurements were carried out using the pHenomal pH 2100L combined with the SF113 stab electrode (VWR, Leuven, Belgium). The a_w was

determined with the AQUALAB 4TE Water Activity Meter (Metergroup, München, Germany). In samples packed under modified atmosphere (MAP), the gas composition was measured at each test moment in an individual blank sample using a headspace gas analyzer (Dansensor Checkmate 3, Erpe-Mere, Belgium), according to the manufacturers' guidelines.

2.2.4 Growth potential calculation

The growth potential was calculated as prescribed in the technical document by taking the difference between the median results at the end and the start of the challenge test. Then the largest value of the difference obtained in three repetitions must be taken as the final value. (CRL EU, 2014). The growth potential in the recently published updated version of the EURL *Lm* Technical Guidance Document is calculated by (1) the highest increase of the Lm enumeration obtained from at least four sampling points when one test unit is analyzed per sampling point or (2) when more than one test unit is analyzed per sampling point, then the highest mean value obtained from each of these four sampling points is taken (CRL EU, 2021). Again, the growth potential retained amongst all tested batches is the highest obtained value.

2.3 Maximum growth rate (μ_{max}) challenge tests in minced pork

A total of three batches were tested (Table 1) in which three replicates were performed each time, namely two inoculated replicates (one per strain used) along with a blank replicate. A pork shoulder (approx. 4.5 kg) was purchased from a local producer on each production day to prepare minced pork. No additives were added to the minced pork used for inoculation and microbiological analysis.

2.3.1 Preparation inoculum, inoculation, and storage

Test units were made in the same way as described above using same packaging materials, with in-house prepared and purchased minced pork at 25g / 375g or 100g / 300g ratios for inoculated and blank test units, respectively. According to the EURL *Lm* Technical Guidance Document (CRL EU, 2014), growth rate challenge tests were performed by monitoring the growth of the two selected strains separately in inoculated series in addition to series of blanks to monitor the microbiome and physico-chemical parameters per batch. The challenge test lasted 14 days, which is much longer than the shelf-

life for both types of minced meat packaging (shelf-life$_{air}$ 2-4 days ; shelf-life$_{MAP}$ 5-8 days (Air Products and Chemicals Inc., 2016)).

As for air-packed inoculated minced meat, *L. monocytogenes* enumerations were carried out on ten times with the daily follow-up the first eight days representing the exponential phase and days 10 and 14. At the same moments physicochemical parameters were measured on blank test units. In addition, the microbiome mentioned previously was determined on blank test units at day 0, 3, 6, 10, and 14.

For MAP, 12 *L. monocytogenes* enumerations were performed for both inoculated series, and physicochemical parameters, including gas composition, were determined for blank test units, the first 10 days and on day 14. The microbiome was determined on blank test units at day 0, 3, 6, 10, and 14.

The inoculum preparation and inoculation were identical to those described for the growth potential challenge tests except that the strains were not combined in equal quantities, but each strain was used individually. All tests were conducted at a fixed temperature of 7°C, mirroring the mean domestic refrigerator temperature (Roccato et al., 2017). Besides, 7°C is a compromised temperature that reflects reality, as it lies between the maximum storage temperature of 4°C for retail minced meat and 9°C, the latter corresponding to the 75th percentile of Belgian domestic refrigerator storage temperatures (CRL EU, 2014; Devriese et al., 2004).

2.3.2 Calculation of maximum growth rate and estimating the growth potential

Growth curves of *L. monocytogenes* enumeration data during the storage period of 14 days at a fixed temperature of 7 °C were used to determine μ_{max} in minced pork for both strains and packaging types. As 3 batches were performed with 2 strains, 6 growth curves could be interpreted, and rates calculated per packaging variant. Growth curves were fitted with the online DMFit tool (available at www.combase.cc) using the Baranyi and Roberts model (Baranyi and Roberts, 1994). The highest growth rate value (log_{10}) for each packaging variant ($\mu_{max,ref}$) was then used to calculate growth rates (μ_{max}) at different temperatures (T) according to the simplified square-root secondary model (Eq. (1)):

$$\mu_{max} = \mu_{max,ref} \cdot \frac{(\mathrm{T} - \mathrm{T}_{min})^2}{(\mathrm{T}_{ref} - \mathrm{T}_{min})^2} \qquad \text{Eq. (1)}$$

with T_{ref} the reference temperature, in our case 7°C, at which the maximum growth rates were obtained, and T_{min} the minimum *L. monocytogenes* growth temperature (−1.5°C) (CRL EU, 2014). At last, the

growth potential at used time-temperature profiles was determined using Eq. (2) (Vermeulen et al., 2011), as suggested by the EU technical guidance:

$$\Delta \mathrm{logCFU/g} = \sum \mu_{max,i} \cdot d_i \qquad \text{Eq. (2)}$$

with Δ log CFU/g the logarithmic development in cell number during the shelf- life, $\mu_{max,i}$ the calculated maximum growth rate from Eq. (1) at a specific temperature (T_i), and d_i the time at temperature T_i.

The calculation as mentioned above does not include a lag phase which is often observed when environmental conditions change (more or less) abruptly. However, the recently updated version of the EURL *Lm* Technical Guidance Document (CRL EU, 2021) mentions using a primary model (Eq. (3)) (Baranyi and Roberts, 1994) to estimate lag times from microbial growth curves:

$$h_0 = \mu_{max} \cdot \lambda \qquad \text{Eq. (3)}$$

allowing calculation of the constant parameter h_0 from which the lag time (λ) at different temperatures was estimated.

In addition, the *L. monocytogenes* growth potential at the used time-temperature profile was obtained using Combase Predictor (www.combase.cc) and DMRI predictive models (www.dmripredict.dk) for meat and compared to the observed growth potential results from the performed challenge test to evaluate the model predictions. Combase is a freely accessible online tool using broth based models for quantitative and predictive food microbiology that studies and predicts the survival and growth of microorganisms under various primarily food-related factors, such as temperature, pH, water activity, and atmosphere composition. The initial mean values from the current experimental setup for the above-mentioned input factors, summarized in Table 4, were used as Combase growth model input factors. The physiological state was set to 1, since the preculture was prepared at the same temperature as the storage experiments, implying that the culture is adjusted and no lag phase is supposed to be occurring (Vermeulen et al., 2011). Models from the Danish predictive safety model for *L. monocytogenes* growth in meat products from the Danish Meat Research Institute (DMRI) are product based models also freely available online and include the effect of temperature, pH, sodium chloride in the water phase, acetic acid/diacetate and lactic acid in the water phase, added sodium nitrite, CO_2 in the packaging atmosphere, and interactions between all these environmental parameters (Mejlholm et al., 2010). The *L. monocytogenes* dynamic temperature safety model was utilized. Only the following parameters were

used as input factors: the average pH from the current experiments, the lowest possible NaCl concentration (0.8%), the water content in the final product (62%) and minced pork was indicated as an emulsified product containing more than contains 10% fat. The storage variables were set to the studied T-profiles. In the case of MAP, 30% CO_2 has been added to the model.

3. Results

In this study, challenge tests were performed on pork chops and minced pork processed from pork carrés to evaluate the behavior of *L. monocytogenes*. When consumed raw, minced pork is regarded as RTE. Initially, the growth potential during shelf-life was determined on three batches for pork chops. Each of the three batches was conducted threefold, providing both intra- and inter-batch variability information for physicochemical and microbial parameters. Besides, determination of growth potential in minced pork was performed in singular as the results exceeded the limit of 0.5 already for this first batch, immediately leading to the (intermediate) conclusion that the prepared minced pork can support the growth of *L. monocytogenes*. Further challenge tests on the maximum growth rate in minced pork were performed accordingly.

All non-inoculated test units, both pork chops and minced pork, showed the absence of *L. monocytogenes*, meaning there was no detectable natural contamination of the processed pork loins and shoulders.

3.1 Growth potential in pork chops

3.1.1 Intrinsic and extrinsic parameters

Mean values of measured physicochemical parameters are presented in Table 2. On day 0, similar values for pH were observed between different batches, around 5.50. Both for the air- and MAP-packed pork chops, results at the end of the shelf-life showed differences between batches, with decreased or stable pH values for batches 1 and 3, while for batch 2 an average pH increase of 0.17 and 0.14 was found for the air-packed and MAP-packed chops, respectively. At the start of the tests, the pork chops' average a_w was 0.997 except for batch 3, which was slightly lower at 0.987. Values for a_w remained stable during the shelf-life for both air and MAP packaging. Also, the atmosphere in MAP-packed pork chops remained stable during shelf-life.

3.1.2 Microbiological analysis

Figure 2 shows the evolution in numbers for *L. monocytogenes* in inoculated pork chops and other microbiota in non-inoculated pork chops at a specified time-temperature profile per packaging type.

In air-packed pork chops, results of non-inoculated samples show comparable low numbers of TAC, Y&M, LAB and *Pseudomonas* at day 0 and 2, while growth of TAC, LAB and *Pseudomonas* especially took place during the last two days of incubation at 9°C. Results also show the inter- and, even intra-batch variability in the microbiome in air-packed pork chops.

With MAP-packed pork chops, TAC was dominated by LAB. Again, intra- and inter-batch variability was observed, especially for LAB.

3.1.3 Growth of L. monocytogenes

The results of the calculated *L. monocytogenes* growth potential in both air-and MAP-packed pork chops are presented in Table 3.

For air-packed pork chops, intra-batch variability for batch 1 was limited compared to batch 2 and 3. In batch 3, there is even one replicate in which the number of *L. monocytogenes* decreased towards the end of shelf-life, while the other replicates showed an increase, confirming both inter- and intra-batch variability.

Likewise, the results for MAP-packed pork chops also showed a clear difference in growth potential between replicates and batches (Table 3). In two replicates, more specifically the first replicate of the second batch for both the air and MAP packaged version, the growth potential value exceeded the limit value of 0.5. Nevertheless, according to the EURL *Lm* Technical Guidance document (CRL EU, 2014) applicable at the moment of investigation in 2020, the growth potential per batch ought be calculated by taking the difference between the median values at the end versus the beginning of the shelf-life, resulting in growth potential values of 0.28 and 0.46, respectively, for the air and MAP-packed 2nd batch.

3.2 Growth potential in minced pork

3.2.1 Intrinsic and extrinsic parameters

Mean values of measured physicochemical parameters at the different time points are presented in Table 4. The pH values at day 0 varied from 5.5 to 5.9. All replicates increased in pH, on average 0.27 in air

and 0.32 in MAP-packed minced meat, respectively. The water activity remained stable with a small standard deviation on the analysis. The atmosphere in MAP-packed minced pork remained unchanged during the shelf-life.

3.2.2 Microbiological analysis

The evolution in microbial numbers at a specified time-temperature profile per packaging type is presented in Figure 3. In air-packed minced meat, the end-of-life TAC was dominated by *Pseudomonas* spp., reaching more than 6 log CFU/g. Microbiota showed significant growth during the last two days of incubation at 9°C. The variability between replicates for the microbiota at the end of the shelf-life was minor, except for noticeably less growth of lactic acid bacteria in replicate 1 (about 3 log CFU/g) in comparison with the other replicates (about 5 log CFU/g). Notable differences in microbial growth were observed for MAP-packed minced meat as well.

3.2.3 Growth of L. monocytogenes

The results of the calculated *L. monocytogenes* growth potential in both air-and MAP-packed minced pork are presented in Table 5. According to action limits (Vermeulen et al., 2011), large (1.99 log CFU/g; between 1.00 and 1.99 imply an absence tolerance in 1 g on day of production) and moderate (0.94 log CFU/g; between 0.50 and 0.99 imply an absence tolerance in 0.1 g on day of production) differences between the growth potential of the three replicates were observed for air- and MAP-packed minced pork, respectively. Limited *L. monocytogenes* growth was found for both packaging variants in replicate 1, while an apparent increase was seen in the other two replicates, especially during the time period at 9°C (Figure 3).

3.3 Maximum growth rate in minced pork

Figure 4 shows the growth curve of the two *L. monocytogenes* strains used and also the microbiome over 14 days at 7 °C for the three batches. These results show that in general similar growth can be observed across the three batches, suggesting a consistent performance of the challenge tests. The air-packed minced pork was dominated by *Pseudomonas* spp. where log 8 was already reached after three days. For MAP-packaged minced meat, lactic acid bacteria was the dominant group. pH values of MAP

minced pork samples initially ranged from 5.8 to 6 and generally remained stable or increased slightly during the first 10 days, with a higher increase in the last 4 days bringing the final values after 14 days between 6.2 and 6.3.

Furthermore, the gas composition in MAP showed a similar course for batch 1 and 2, with the CO_2 fraction increasing from day five up to 70% at the end of the tests. For batch 3, the proportion of CO_2 steadily increased from the start to 50% at the end of the tests.

Fitting complete or no lag Baranyi models in Combase gave restricted or even non-existing lag phases and resulted in high correlation coefficients for each growth curve and small standard deviations on the estimated μ_{max} (Table 6). The highest μ_{max} obtained from the six growth curves at 7 °C was set at 0.680 and 0.489 log CFU/day for air-packed and MAP minced pork, respectively.

From these μ_{max}, the growth rate at used time-temperature profiles was calculated using a square root secondary model as recommended by the EU guidance document (Table 7). Air-packed minced meat kept 2 days at 4 °C (storage at retail) and 2 days at 9 °C (storage in a domestic refrigerator) resulted in a maximum increase of 2.64 log CFU/g (0.28 × 2 + 1.04 × 2). A maximum increase of 2.52 log CFU/g (0.20 × 5 + 0.75 × 2) was obtained for MAP minced pork kept 5 days at 4°C and 2 days at 9°C. Moreover, the growth potential was predicted using the Combase growth model and the DMRI dynamic safety model.

Figure 5 compares the growth potential observations from the minced pork experiment with the calculated growth potential values from the analyzed data and models. The predicted growth potential values show an overestimation of less than 0.5 log CFU/g on the intermediate day in air-packed minced pork. At the end of the shelf-life, most model predictions fall within the growth potential range obtained from the experiments, except for the square root model, where the lag phase was not considered (CRL EU, 2014). In MAP packed minced meat, the differences are more prominent between the experimentally obtained growth potential values and the model predictions, ranging from a 0.7 log CFU/g (DMRI dynamic safety model) to 1.7 log CFU/g overestimation (square root model, EURL, 2014) at the end of the shelf-life.

4. Discussion

The EURL *Lm* Technical Guidance Document of 2014 for conducting shelf-life studies on *L. monocytogenes* in ready-to-eat foods was used in this study. More specifically, challenge tests were performed on pork chops and minced pork. Results showed that (i) pork chops did not support the growth of *L. monocytogenes* under the used storage conditions throughout its shelf-life, implying minimal risk; (ii) minced pork showed apparent growth for both packaging types; and (iii) the obtained growth rate was higher in air than in MAP-packaged minced pork.

4.1 Growth potential challenge tests

4.1.1 Pork chops do not support L. monocytogenes growth

Since the regularization (EC no 2073/2005) regarding the microbiological limits refers explicitly to ready-to-eat (RTE) products, few studies have been conducted on the prevalence, concentrations, and especially growth of *L. monocytogenes* in fresh pork cuts. *L. monocytogenes* prevalence of 10% in raw pork products at retail and 3% in ready-to-eat pork products were found in literature and used for pork risk profile calculations (Mataragas et al., 2008). A Finnish study found a prevalence of 4% on raw pork cuts (Hellström et al., 2010). In Italy, 10% of raw pork samples were found *L. monocytogenes* positive (Busani et al., 2005). *L. monocytogenes* was found in 24% of raw pork chops from Canadian retail marketplaces (Bohaychuk et al., 2006). Although present on raw pork cuts, limited literature was found on the level of initial natural contamination of pork chops, which is needed to improve risk allocation. A study by De Cesare et al. (2014) tested the pathogen's presence and concentration, showing a substantial percentage of positive samples with counts lower than 10 CFU/g in 44% and a mean concentration of 2.02 log CFU/g in 5% of the tested pork chops immediately after packaging.

To our knowledge, the growth potential of this pathogen in pork chops has not been described in previous studies. From the present study's results, it appeared that pork chops did not support the growth of *L. monocytogenes* under the foreseen temperature profiles, with growth potential values of respectively 0.28 and 0.46, for air and MAP. However, significant intra-batch variabilities were observed, which were not considered as the protocol described taking the difference between the median values, omitting the extremes, which leads to (potential) underestimations. For example, the first replicate of the second batch gave the highest growth potential for both the air and the MAP packaged

chops, with a value of 0.77 and 1.26, respectively, both above the cut-off value of 0.5, implying the potential supported growth of the pathogen. Therefore, we hypothesize that, based on the precautionary principle, it would be better to assess the growth potential of the batch by taking the highest increase in *L. monocytogenes* numbers of the three individual replicates rather than the difference between the median values of the three replicates at the end versus the beginning of shelf-life, as already pointed out earlier (Vermeulen et al., 2011). ANSES recently published the updated new version of the EURL *Lm* Technical Guidance Document, which takes the highest batch growth obtained, taking into account each sampling point in time, to calculate the growth potential (CRL EU, 2021). The average considers extreme values resulting in higher growth potential for batch 2 and which would be exceeding the 0.5 value for MAP-packed pork chops. At the same time, one should remain critical because one replica in batch 2 looks like an unexpected value (scrutinization of experimental setup and lab notes could not explain this observation)

Both intra- and inter-batch variability in growth potential illustrated differences in pathogen behavior likely related to chop heterogeneity as batch replicates were taken from different animals. The pH and a_w values between replicates and batches were similar and remained relatively constant throughout the test period for both packaging types. The difference in *L. monocytogens* behavior might be attributed to interaction with the notably variable competing microbiome, indicating the complexity of the microbial ecosystem and its metabolites on raw meat surfaces (Roberts et al., 2005). As well, the complex working spectrum of lactates and lactic acid in the meat postmortem resulting from anaerobic glycolysis may have inhibited *L. monocytogenes* growth and thus played a role in the obtained variability. In this context, the animal's history, being PSE (Pale, Soft, and Exudative) or DFD (Dark, Firm, and Dry) meat, as well as the pH which determines the dissociation of lactic acid, would influence the growth of *L. monocytogenes* (Guo et al., 2021; PURAC et al., 1998; Scheffler et al., 2013, 2011). Measurement of lactic acid would have been useful in this context. However, to our knowledge, there is no literature available demonstrating the effect of natural lactate accumulation in the pork postmortem having an inhibitory effect on the growth of *L. monocytogenes*. Studies describing the amount of lactate postmortem in the pork longissimus muscle indicate an amount of 72 µmol/g (Hambrecht et al., 2005). For PSE meat, a value of 111 µmol/g was obtained in the same muscle (Van Laack and Kauffman, 1999). These concentrations are considerably lower than the minimum inhibitory concentrations

determined for sodium lactate, which vary from 410 µmol/g to 800 µmol/g (Houtsma, 1996). Still, further research is needed to determine the role of accumulated lactate naturally present in the animals' tissue on the behavior of *L. monocytogenes*.

Moreover, growth is mainly a function of storage temperature and storage time, suggesting the importance of the initial and intermediate microbiota balance and numbers on the meat surface and explaining the divergent growth. Therefore, a deeper analysis of the impact of the microbiome will be discussed further in the challenge tests performed at a constant temperature part (4.3).

Despite that, as described above, growth appeared possible in some replicates, and levels may already be countable from the beginning in a few cases (Demaître et al., 2020). Still, it may generally be hypothesized that *L. monocytogenes* will only rarely exceed 2 log CFU/g in naturally contaminated raw stored loin chops at refrigeration temperature during their shelf-life. However, abuse of refrigerator temperatures by consumers may allow concentrations of at least 3 log CFU/g to be reached, indicating the need for a rather severe heat inactivation treatment (De Cesare et al., 2013). Different cooking treatments demonstrated a reduction between 6 and 7 log CFU/g of *L. monocytogenes*, and in addition, simulations showed that the few cells surviving cooking treatments might multiply during storage by consumers up to 1 log CFU/g (De Cesare et al., 2018).

Regardless of the low numbers and limited growth, cross-contamination from raw meat to other raw or cooked foods remains a significant risk in the domestic environment, highlighting the importance of consumer attitude and awareness towards food preparation practices, such as avoidance of cross-contamination; adequate refrigeration; proper cooking; adequate post-cooking handling (ILSI Research Foundation; Risk Science Institute, 2005; Nørrung and Buncic, 2008). According to the National Food Consumption Survey, only 10% of the Belgian population adopts a correct attitude when touching raw meat to avoid different ways of cross-contamination (De Ridder et al., 2016).

4.1.2 Growth potential is present in minced pork

About 20% of the minced pork samples were contaminated with the pathogen in Japan (Inoue et al., 2000). Logically, minced pork is more likely to contain pathogens than pork cuts since grinding meat involves more handling steps and processing of the meat and subsequently provides additional occasions for contamination. Apart from a foreseen higher prevalence, the pathogen count is expected to be low

in most cases, estimated at 14-17 CFU/kg in minced pork based on presence/absence data using a Bayesian modeling approach (Andritsos et al., 2013), and thus challenging to count by direct plating.

The present study results showed the pathogen's well-supported growth in minced pork after performing only one batch under reasonably foreseen temperature profiles, with growth potential values of respectively 1.69 and 0.80, for air and MAP. However, when the average is taken to calculate the growth potential, as described in the updated new version of the EURL *Lm* Technical Guidance Document (CRL EU, 2021), the value drops below 0.5 for MAP-packed minced pork. The negative value seen in replica 1, for which we could not find the underlying reason, causes this reduced value, which confirms the sensitivity of this central tendency measure to extreme values. There are, of course, only 3 values for determining the median or average, so using these measures of central tendency with the observed intra-batch variability can give inconsistent results. Also, in the case of minced pork, only 1 batch was examined.

It should be noted that in the present study, pure minced pork has been used without any additives, not even with sodium chloride, which is naturally found in low concentrations (100 mg/100 g; 0.10%), and also without organic acid salts, which are generally used to control and limit the growth of pathogens due to their ability to lower pH and a_w (Zuliani et al., 2007). The water activity continually showed permissive *L. monocytogenes* growth values (>0.990), and the observed increasing pH may also have favored pathogen growth. Growth may also be attributed to inoculation causing the pathogen to become a more dominant microbiota at time 0, thus having a competitive advantage, which is probably less common in naturally contaminated foods (Eicher et al., 2020). However, unlike the other two replicates, the first replicate did not support pathogen growth (δ_{air}: 0.24; δ_{MAP}: -0.13), demonstrating intra-batch variability. Again, the microbial balance and numbers at the intermediate temperature shift and the adaptability to the changing temperature may determine further growth of specific microbiota and possibly explain the divergent growth observed. It is established that LAB characteristics and levels may vary and influence the interaction with *L. monocytogenes* and the pathogen's growth opportunities (Uyttendaele et al., 2004). For the two replicates supporting pathogen growth, rapid growth was only observed at 9°C, representing a food safety risk associated with household refrigeration practices.

4.1.3 Reflections regarding performed growth potential challenge tests

The challenge tests performed revealed the pathogen's behavior in raw pork. Even though pork chops are not a RTE product and air-packed minced pork is classified as unable to support the growth of *L. monocytogenes* (based on its shelf-life shorter than five days), the results can be further used to refine previous risk assessments and define control measures by the meat industry and retail. These concerns are primarily related to the continued retail pressure experienced by the sector over the presence of *L. monocytogenes* on incoming carcasses, meat, and meat products.

Besides, we would like to point out that the obtained results can also provide a critical look at the basic design of the compromise-based protocol. Our results show how challenging it is to establish opportunities for *L. monocytogenes* growth in pork chops. Given the possible heterogeneity in these products, when estimating the growth potential with challenge tests, there remains a certain residual chance that growth in other batches with similar properties is underestimated or overestimated. Therefore, it is recommended to specify or adapt the method of performing challenge tests in such heterogeneous products, as is also the case for fermented foods, for example, in the technical guidance document. One batch does not represent a product whose intrinsic properties are very similar to such products, so we suggest performing single replicate challenge tests with a more significant number of batches.

Furthermore, product-specific parameters affecting the pathogen's behavior should be thoroughly investigated and followed at regular time points. For example, the lactate concentrations in fresh meat, whether of natural origin or added, may affect *L. monocytogenes*. In this context, it would also have been interesting to investigate extrema, i.e., PSE and DFD pork, and the influence on the behavior of *L. monocytogenes*.

Before setting up challenge tests, durability studies may be performed to determine the variability of representative physicochemical characteristics of the (meat) product at the start of the planned storage period to help understand the product's typical (bio)chemical and microbial (spoilage) processes developing during storage. This preliminary study requires many tests of naturally contaminated batches but can contribute to further validations.

From a scientific point of view, inoculation of single strains would be interesting to investigate their growth characteristics and strain variability, which is needed to assess their growth potential fully.

4.2 Maximum growth rate challenge tests

4.2.1 Growth rate in minced pork at 7°C

From challenge tests performed at a constant temperature of 7°C, maximum growth rates were assessed using the Baranyi and Roberts model (Baranyi and Roberts, 1994). Negligible inter-batch variability was observed for growth rate tests conducted in both packaging types, indicating consistent behavior of *L. monocytogenes* and the microbiome in minced pork at constant temperature and proving uniformity in the performance of the replicate testing. Growth curves of the reference strain showed limited to no lag phase, while our strain showed an albeit short lag phase in most cases (Figure 4, Table 6). This is likely due to the use of cold-adapted strains, which have been shown to have a significant effect (Uyttendaele et al., 2004). Pre-incubation at 7°C allows adaptation of the strains and thus partly justifies this storage temperature during the tests. In addition, 7°C was also the most frequently mentioned maximum refrigerator temperature in Belgium, although considerable variation may be expected, and is close to the 8°C referred to in several studies (Roccato et al., 2017; Vermeulen et al., 2011).

The shelf-life of the minced pork, defined as the time in which the dominant spoilage genera, determined by the storage conditions, reach 10^7 CFU/g (BIOHAZ, 2016; Mano et al., 1995), was exceeded far before the end of the challenge tests, approximately after 2 days under air and 4 days under MAP. These results confirm the rapid growth of specific spoilage organisms and *L. monocytogenes* in minced pork at a temperature of 7°C. *Pseudomonas* spp. are recognized as significant spoilage agents on fresh meat and thus fresh minced pork stored under aerobic conditions (BIOHAZ, 2016; Koutsoumanis et al., 2006; Papadopoulou et al., 2020), which was also reflected in our results, with numbers reaching up to 10 log CFU/g after 6 to 9 days of storage. In addition, an eloquent pH increase of almost 2 was observed from day 4, which is probably due to the high numbers of spoilage organisms and may promote the growth of *L. monocytogenes* (Mano et al., 1995). *L. monocytogenes* numbers reached their maximum population density (MPD) at 7 log CFU/g after approximately 10 to 12 days in air-packed minced pork. No impression of adapted pathogen growth due to microbial interactions was observed as both *L. monocytogenes* strains continued to grow while the dominant *Pseudomonas* spp. had already reached their maximum, suggesting the absence of the Jameson effect and virtually independent growth (Buchanan and Bagi, 1997).

In MAP, it is well understood that the antimicrobial activity of CO_2 in the headspace inhibits microbial growth, primarily of *Pseudomonas* spp., resulting in a shift in the dominant microbiota towards bacterial groups with less spoilage potential, extending the shelf-life (Koutsoumanis et al., 2008; Nieminen et al., 2015). This is evidenced by our results in which the lactic acid bacteria became the major spoilage organisms in MAP minced pork, typically reaching maximum numbers of 8 log CFU/g, generally characterized by souring rather than putrefaction (BIOHAZ, 2016). Although a decrease in pH due to lactic acid production would be expected associated with an increasing percentage of Gram-positives in the total microbiota (Nassos et al., 1983), the pH in this study remained constant or even tended to increase very slightly during the first 10 days and certainly during the last 4 days. The oxygen-rich MAP in the current study might, for example, have selected *Leuconostoc gelidum* subsp. *gasicomitatum* over other psychrotrophic LAB strain communities whose respiratory metabolism produces acetoin and diacetyl as end products and limits the acidification of the minced pork (Nieminen et al., 2015). *L. monocytogenes* growth is inhibited in the presence of LAB, including *Leuconostoc gelidum* spp., partly due to bacteriocins production and in some cases also due to a potentially significant pH effect (Griffiths, 2003). In general, *L. monocytogenes* growth decelerated after 9-11 days at 5 log CFU/g, when LAB reached their maximum, corresponding to the so-called Jameson effect. In that case, microbial interactions may have reduced the pathogen's maximum population density. Additionally, the pathogen's growth may be inhibited by the presence of carbon dioxide in the packaging atmosphere (Saraiva et al., 2016). It was also observed that the variation was more considerable between the growth curves of the different batches and the two strains used for MAP minced pork. Of course, growth curves were constructed from data points derived from individual samples, which may imply a sequence of variability in microbial communities and thus influence the fit, especially at the beginning of the growth curve. As always, by doing such a fit, it is hoped that a kind of average behavior is described.

4.2.2 Predicting L. monocytogenes *growth in minced pork based on models*

The accuracy of model predictions appeared to depend mainly on the packaging used, although evident differences between models were also observed.

As for air-packed minced pork, the calculated growth potential for the studied T-profiles (2 d at 4°C, 2 d at 9°C; $\delta = 2.64$), obtained from the square root model, was considerably higher at the end of shelf-

life than the actual data realized experimentally (δ_{air} = 1.69). Although maximum growth rate challenge tests proved highly beneficial in terms of flexibility, as there is the possibility to extrapolate growth rates at different temperatures (below 25°C) in the same food, the predictions of growth potential by the said square root model seem to overestimate reality, especially at the end of shelf-life. It is conceivable that the interaction effects with the microbiome could lead to the overestimation of the growth potential, as these effects are not considered in the extrapolation and yet expected to be very diverse at different temperatures. The question can be raised whether the application in which a model that takes into account the microbiome, which would be technically more difficult, could be implemented in the EURL technical document. On the other hand, the predictions may in this way be considered 'worst case' and fail-safe as raw ground pork is consumed raw or undercooked. Still, as always, a model that is too fail-safe becomes ethically and economically irrelevant when it comes to applications (McKellar and Lu, 2003). Additionally, the square root model does not consider the lag phase, which may be acknowledged as a crucial parameter for a product with a short shelf-life, such as minced meat. The predictions more closely approximated the experimental values (within 0.5 log CFU/g) by applying the primary model to estimate lag phases and take these into account, as indicated in the new protocol (CRL EU, 2021). Of course, the assumption in this model (that lag times for identical inocula introduced to environmental conditions are inversely proportionate with growth rates) has limits and is probably most applicable to changes in temperature (McKellar and Lu, 2003). An explanation that this latter model, incorporating the lag phase, still gave an, albeit less, overestimation might lie in a longer lag phase duration for temperature shifts outside the normal physiological temperature range (NPTR), which would require additional work to adapt to a new environment (McKellar and Lu, 2003). However, a study of Xanthiakos et al. (2006) demonstrated accurate predictions regarding *L. monocytogenes* behavior in pasteurized milk under dynamic temperature conditions, between 4°C and 12°C, implying the temperature range used in our study probably extends within the microorganism's NPTR. Also, the Combase and the DMRI models give good predictions of the growth potential values obtained from the performed tests (within 0.5 log CFU/g) for air packed minced pork.

In MAP minced pork, all models considerably overestimated (up to more than 1 log CFU/g) the growth potential values obtained from the actual data realized experimentally. These overestimations can mainly be attributed to the lack of integration of the carbon dioxide effect in the models used. As can be

seen from Figure 5, the headspace carbon dioxide changes due to, among other things, the gas solubility in the minced pork, the carbon dioxide dissociation, and microbial respiration, which will inevitably affect both the metabolism and energetics of *L. monocytogenes* cells and thus its growth rate. Also, the inhibitory effect of carbon dioxide on the growth of *L. monocytogenes* appeared to be greater at lower temperatures (8 and 12 °C) due to a higher solubility (Couvert et al., 2017). For example, predictions from the Combase growth model only calculate the possible inhibitory effect of a constant CO_2 concentration in the headspace but do not consider CO_2 gradients in food or CO_2 transfer through the packaging material (Chaix et al., 2015). However, in this model, we also dictated optimal adaptation of the pre-cultured cells, suggesting that no lag phase would occur, which is certainly not the case given the adaptations to the modified atmosphere. Modifying the physiological state in the Combase growth model can make the prediction more accurate, although this parameter is uncertain and difficult to estimate and the prediction is then intertwined with some fitting efforts. DMRI's predictions were noticeably better and closer to reality. The DMRI dynamic safety model, which is developed explicitly for freshly produced RTE meat products (pasteurized to a core temperature of 75 °C and cured/dried), incorporates the most intrinsic and extrinsic factors, including CO_2, and also considers their interactions. It remains a fact that *L. monocytogenes* growth on food matrix, especially raw meat products, is notoriously difficult to predict or model due to varying intrinsic and extrinsic factors as well as interaction effects with the microbiome. In addition, in MAP, the dynamics of gas exchanges in the packaging and its environment and the effect on microbial growth should also be considered.

4.2.3 Reflections regarding predictive modelling in raw pork

The highly variable microbial growth in shelf-unstable food, such as raw pork cuts and minced pork, questions the usefulness of predicting *L. monocytogenes* growth based on models for these products. Modeling approaches, particularly in MAP, overestimated the pathogen's cell count observed in the growth potential challenge tests. In addition, model predictions indicated the lack of integration of intrinsic and extrinsic factors, interaction, and atmosphere effects and rendered these models inadequate to predict the growth of *L. monocytogenes* in raw minced pork with sufficient accuracy. Predictive tools can still improve by further integrating time-dependent intrinsic and extrinsic factors, interactions, and

especially dynamic atmosphere effects could potentially increase the accuracy of predictions. Also, a product-based model can be strived for, with the option of integrating margins of error.

Our findings demonstrate that using single-point risk assessment calculated estimates for these raw meat products is far from reality, given the variability obtained from experimental tests. Instead, a probabilistic approach through a quantitative microbiological risk assessment (QMRA), with simulation scenarios of the time-temperature evolution of raw pork along the cold chain, might be a better way to estimate these raw meat products' food safety.

5. Conclusion

Pork chops might be considered a lower risk product as no sustained growth of *L. monocytogenes* was observed for air or MAP, at least for the specific conditions used in this study. Besides, pork chops are typically heated extensively before consumption, which kills the pathogen. Still, the risk of cross-contamination with these pork products must be considered, with *L. monocytogenes* growing to high levels under refrigerated abuse conditions and potentially being transferred to other foods and surfaces due to a lack of proper food preparation practices. The obtained results can be used in the future as representative for model studies into the possible outgrowth on pig carcasses. The substantial growth of *L. monocytogenes* (>0.5 log CFU/g) was obtained in pure minced pork for air and MAP, implying a food safety risk associated, especially at abused household refrigeration practices. The observed inter- and intra-batch variabilities when performing these experiments confirmed the complex impact of intrinsic and extrinsic factors on the growth of *L. monocytogenes* in and on raw pork. In addition, calculating the growth potential using the recently published protocol gave a different outcome in MAP and confirmed the sensitivity of using measures of central tendency for these tests, leading to under-and overestimations.

Extrapolating with the obtained maximum growth rates and using existing models (Combase growth model and DMRI dynamic safety model) gave overestimations of the *L. monocytogenes* cell count obtained in the growth potential challenge tests, especially in MAP. Consequently, the predictions and models used are lacking to include the main intrinsic and extrinsic factors specific to raw meat products and the interaction effects with the microbiome and modified atmospheres. Particular knowledge and caution in the use of these predictive models are therefore assuredly required.

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TABLES

Table 1. Overview of the conducted challenge tests.

Product	Challenge test	Time - temperature profile	Number of batches performed	Number of replicates per batch		Units per replicate in time (days)															Total number of units per batch
						0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Air-packed pork chops	δ	2 d at 4°C; 2 d at 9°C	3	Inoculated	3	◍		◍		◍											
				Non-inoculated	3	●		●		●											
MAP-packed pork chops	δ	6 d at 4°C; 4 d at 9°C	3	Inoculated	3	◍						◍				◍					
				Non-inoculated	3	●						●				●					
Air-packed minced pork	δ	2 d at 4°C; 2 d at 9°C	1	Inoculated	3	◍		◍		◍											
				Non-inoculated	3	●		●		●											
	μ_{max}	14 d at 7°C	3	Inoculated	2	◍	◍	◍	◍	◍	◍	◍	◍			◍				◍	2
				Non-inoculated	1	●	◒	◒	●	◒	◒	●	◒			●				●	1
MAP-packed minced pork	δ	5 d at 4°C; 2 d at 9°C	1	Inoculated	3	◍					◍		◍								
				Non-inoculated	3	●					●		●								
	μ_{max}	14 d at 7°C	3	Inoculated	2	◍	◍	◍	◍	◍	◍	◍	◍	◍	◍	◍				◍	2
				Non-inoculated	1	●	◒	◒	●	◒	◒	●	◒	◒	◒	●				●	1

◍: *L. monocytogenes* inoculated test units; ●:blanc test units for determining the microbiome, pH, A_w (and gas composition); ◒:blanc test units for determining pH, A_w (and gas composition). Test units to check natural *L. monocytogenes* contamination are not shown.

Table 2. Mean and standard deviation of measured physico-chemical parameters during the shelf-life period in pork chops.

Packaging	Parameter	Batch	Day_0	Day_{inter}*	Day_{end}*
Air	pH	1	5.49 ± 0.03	5.60 ± 0.05	5.49 ± 0.05
		2	5.45 ± 0.07	5.53 ± 0.05	5.62 ± 0.06
		3	5.55 ± 0.06	5.46 ± 0.03	5.44 ± 0.04
	a_w	1	0.997 ± 0.001	0.995 ± 0.001	0.996 ± 0.002
		2	0.997 ± 0.003	0.995 ± 0.004	0.992 ± 0.004
		3	0.987 ± 0.001	0.986 ± 0.000	0.987 ± 0.001
MAP	pH	1	5.49 ± 0.03	5.42 ± 0.03	5.44 ± 0.05
		2	5.45 ± 0.07	5.51 ± 0.07	5.59 ± 0.08
		3	5.55 ± 0.06	5.49 ± 0.04	5.41 ± 0.04
	a_w	1	0.997 ± 0.001		0.992 ± 0.005
		2	0.997 ± 0.003	0.994 ± 0.001	0.992 ± 0.001
		3	0.987 ± 0.001	0.985 ± 0.001	0.987 ± 0.000
	O_2 - CO_2	1	68.5 – 26.3	70.4 - 24.2	69.7 - 25.8
		2	68.3 – 24.8	68.3 - 24.2	67.4 - 24.3
		3	69.6 – 25.4	72.6 - 22.5	70.5 - 23.1

*Days are according to the T-profile: Air: Day_{inter} after 2 days storage at 4°C, Day_{end} after another 2 days storage at 9°C for air-packed chops and MAP: Day_{inter} after 6 days storage at 4°C, Day_{end} after another 4 days storage at 9°C for MAP chops.

Table 3. Calculations of growth potential per replicate and per batch (log CFU/g) in pork chops.

Product	Packaging	Sample	Day_0	Day_{inter}*	Day_{end}*	$\delta_{per\ replicate}$	$\delta_{per\ batch}$ (2014)	$\delta_{per\ batch}$ (2021)
Pork chops	Air	1.1	2.60	2.04	2.04	-0.56		
		1.2	2.60	2.28	2.10	-0.50	-0.56	-0.56
		1.3	2.53	2.07	1.91	-0.61		
		2.1	2.11	1.85	2.88	**0.77**		
		2.2	1.90	1.67	2.19	0.28	**0.28**	**0.49**
		2.3	1.70	1.78	2.12	0.43		
		3.1	2.02	1.70	1.68	-0.34		
		3.2	1.72	1.89	2.03	0.31	0.02	0.09
		3.3	2.01	1.65	2.30	0.29		
	MAP	1.1	2.60	1.80	1.96	-0.64		
		1.2	2.60	1.86	1.86	-0.74	-0.64	-0.57
		1.3	2.53	2.00	2.19	-0.34		
		2.1	2.11	2.40	3.37	**1.26**		
		2.2	1.90	1.90	2.36	0.46	**0.46**	**0.69**
		2.3	1.70	1.78	2.04	0.34		
		3.1	2.02	1.84	1.49	-0.52		
		3.2	1.72	2.02	1.98	0.26	-0.52	-0.28
		3.3	2.01	1.50	1.42	-0.59		

*Days are according to the T-profile: Air: Day_{inter} after 2 days storage at 4°C, Day_{end} after another 2 days storage at 9°C for air-packed chops and MAP: Day_{inter} after 6 days storage at 4°C, Day_{end} after another 4 days storage at 9°C for MAP chops. $\delta_{per\ batch}$ (2014) = difference between the median of the

end minus the median of the initial. $\delta_{per\ batch}$ (2021) = average at the end minus the average of the initial.

Table 4. Mean and standard deviation of measured physicochemical parameters at different time points during the shelf-life period in minced pork.

Packaging	Parameter	Batch	Day_0	Day_{inter}*	Day_{end}*
Air	pH	1	5.69 ± 0.22	5.93 ± 0.08	5.96 ± 0.31
	a_w	1	0.997 ± 0.000	0.995 ± 0.002	0.993 ± 0.002
MAP	pH	1	5.69 ± 0.22	5.93 ± 0.28	6.01 ± 0.34
	a_w	1	0.997 ± 0.000	0.995 ± 0.001	0.993 ± 0.001
	O_2 - CO_2	1	68.5 - 26.3	72.57 - 22.3	69.2 - 25.6

* Days are according to the T-profile: Air: Day_{inter} after 2 days storage at 4°C, Day_{end} after another 2 days storage at 9°C for air-packed chops and MAP: Day_{inter} after 5 days storage at 4°C, Day_{end} after another 2 days storage at 9°C for MAP chops.

Table 5. Calculations of growth potential per replicate and per batch (log CFU/g) in minced pork.

Product	Packaging	Sample	Day_0	Day_{inter}*	Day_{end}*	$\delta_{per\ replicate}$	$\delta_{per\ batch}$ (2014)	$\delta_{per\ batch}$ (2021)
Minced	Air	1.1	2.24	2.36	2.48	0.24		
		1.2	2.56	2.42	4.79	**2.23**	**1.69**	**1.39**
		1.3	2.30	2.16	3.99	1.69		
	MAP	1.1	2.24	2.34	2.10	-0.13		
		1.2	2.56	2.36	3.27	0.71	**0.80**	**0.46**
		1.3	2.30	2.07	3.10	**0.80**		

* Days are according to the T-profile: Air: Day_{inter} after 2 days storage at 4°C, Day_{end} after another 2 days storage at 9°C for air-packed chops and MAP: Day_{inter} after 5 days storage at 4°C, Day_{end} after another 2 days storage at 9°C for MAP chops. $\delta_{per\ batch}$ (2021) = growth potential determined based on new version of EURL Guideline.

Table 6. Growth rates performed by Baranyi and Roberts models using Combase DMFit.

Packaging	Strain	Batch	μ_{max} (log_{10} CFU/g/day)	STDEV	R^2	Lag time (days)	N_{max} (log_{10} CFU/g)
Air	1hLM	1	0.653	0.0697	0.981	1.727	6.927
		2	0.626	0.0654	0.983	1.004	6.745
		3	0.609	0.0605	0.983	1.033	6.397
	12MOB085	1	0.525	0.0184	0.995		6.372
		2	0.680	0.112	0.953	0.445	6.522
		3	0.442	0.0429	0.982	0.407	6.767
MAP	1hLM	1	0.397	0.0453	0.980	2.043	4.675
		2	0.475	0.0617	0.964	2.164	5.266
		3	0.477	0.0358	0.979		5.322
	12MOB085	1	0.309	0.0353	0.949		3.819
		2	0.433	0.0374	0.985	0.546	4.781
		3	0.489	0.0663	0.97	0.261	5.166

Table 7. Increase in *L. monocytogenes* (log CFU/g) predicted by the different models for the studied T-profile and the increase obtained from the challenge tests to assess the growth potential.

		Air		MAP	
		Day_{inter}	Day_{end}	Day_{inter}	Day_{end}
Growth potential		0.06	1.69	0.04	0.80
Square root model	(EURL 2014)	0.57	2.64	1.02	2.52
	Including lag time (EURL 2021)	0.27	2.04	0.90	2.26
Combase growth model		0.58	2.07	1.08	2.27
DMRI dynamic safety model		0.38	1.91	0.48	1.46

FIGURES

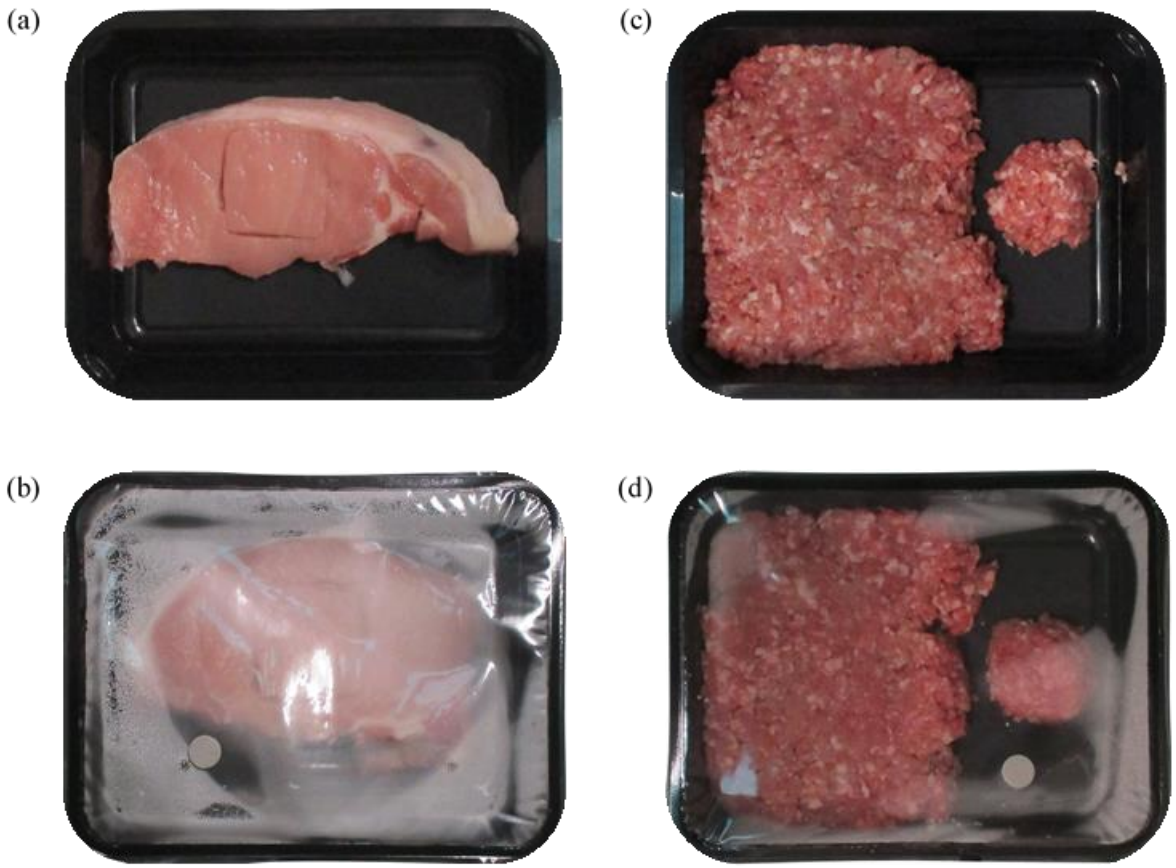


Figure 1. Setup of meat dishes and delineated area in pork chops. (a) air-packed and (b) MAP-packed pork chops; (c) air-packed and (d) MAP-packed minced pork (375g/25g ratio for inoculated test units; 300g/100g for non-inoculated test units)

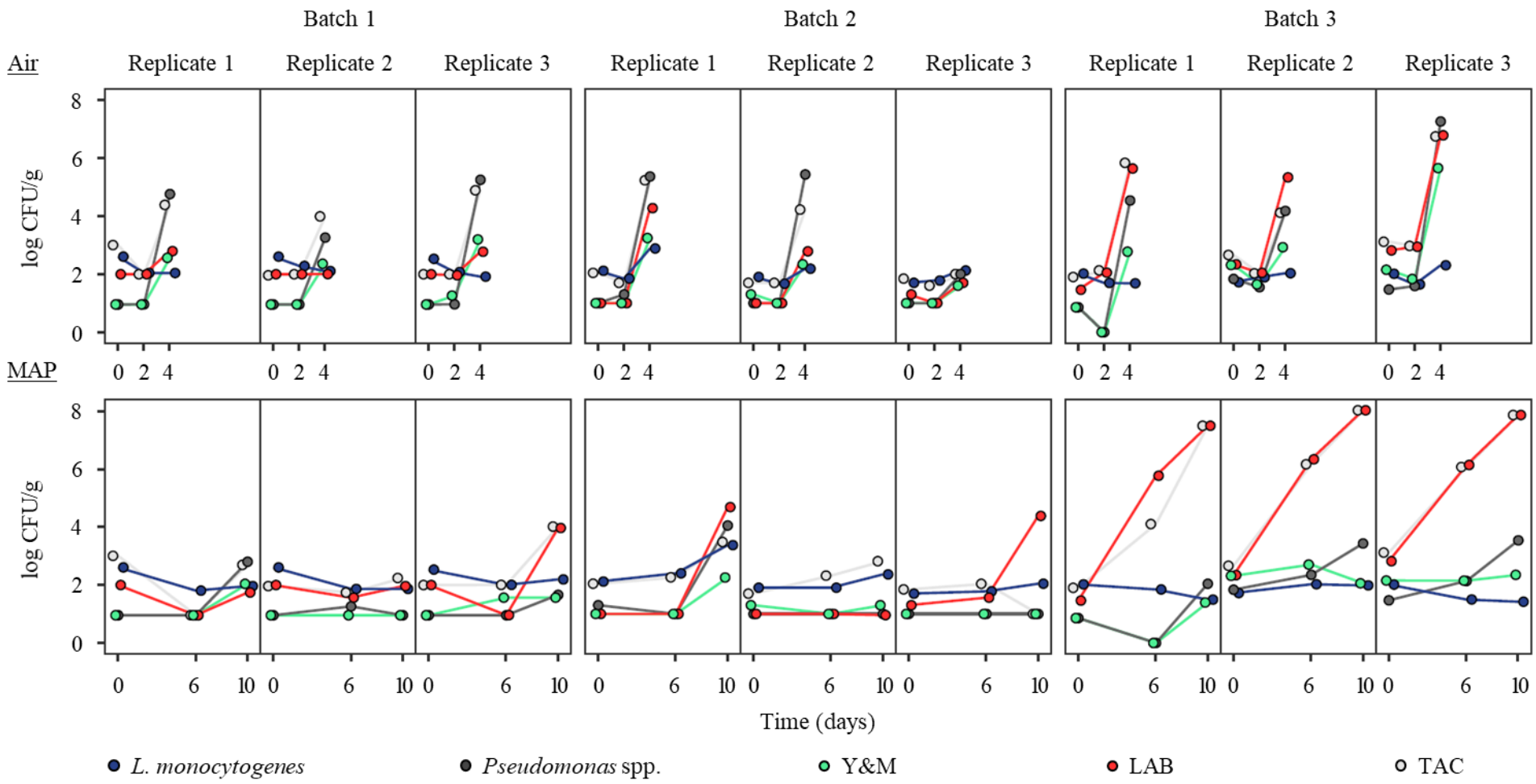


Figure 2. Evolution in microbial numbers for *L. monocytogenes*, *Pseudomonas*, yeast and molds (Y&M), psychrotropic lactic acid bacteria (LAB), and total aerobic psychrotropic count (TAC) per packaging type in pork chops. See Table 1 for storage conditions.

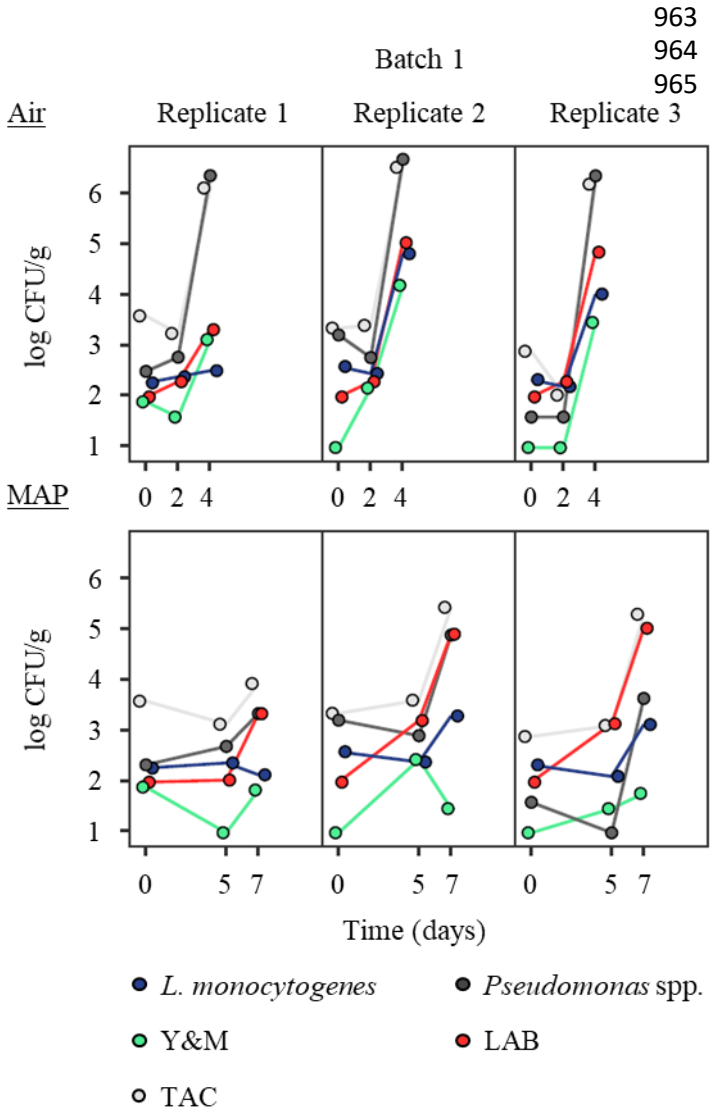


Figure 3. Evolution in microbial numbers for *L. monocytogenes*, *Pseudomonas*, yeast and molds (Y&M), psychrotropic lactic acd bacteria (LAB), and total aerobic psychrotropic count (TAC) per packaging type in minced pork. See Table 1 for storage conditions.

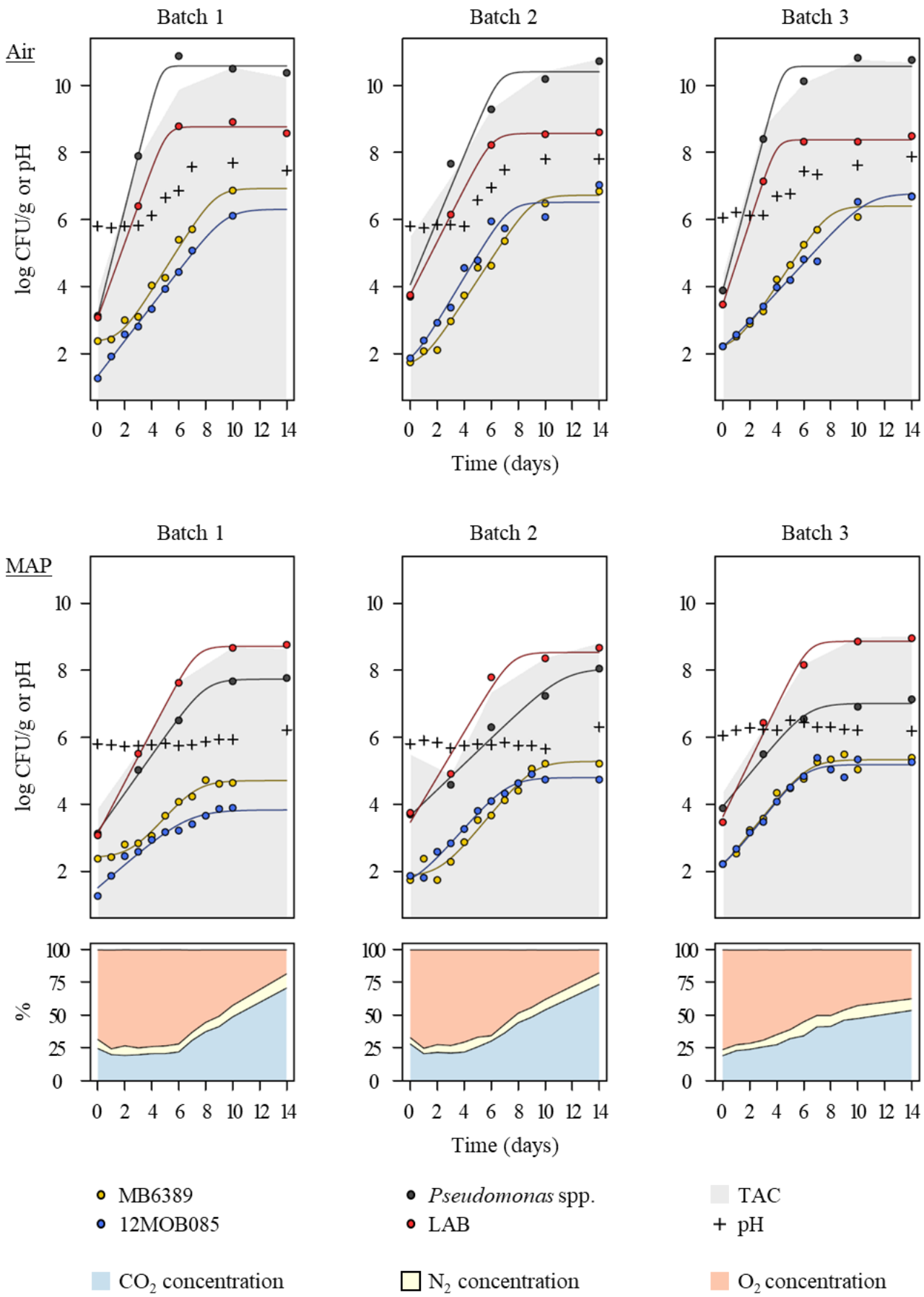
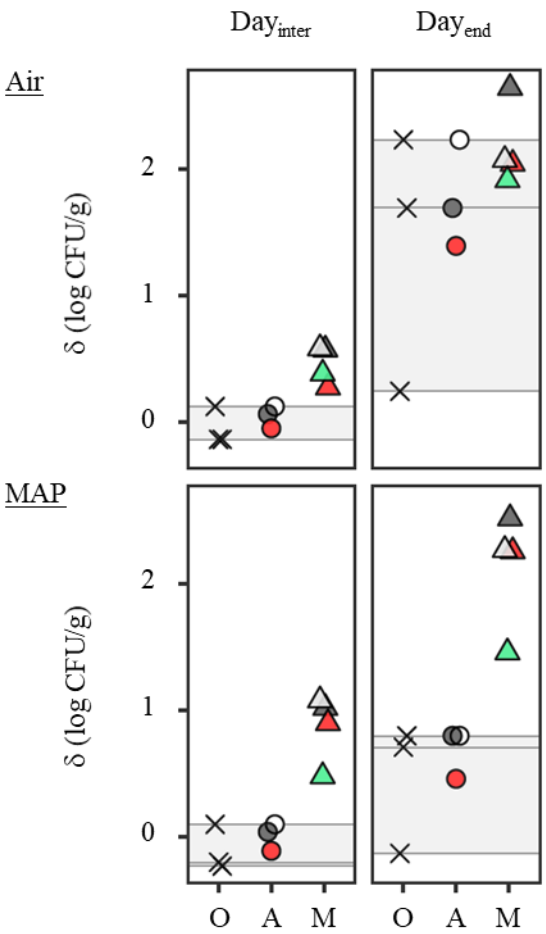


Figure 4. Evolution of *L. monocytogenes*, the dominant microbiome and pH at 7°C in minced pork per packaging type and gas composition evolution in MAP. Lines represent the fitted complete or no lag phase Baranyi growth curves.



- O (**Observations**): represented by crosses and showing the experimental observed growth potential of the three replicates.

- A (**Data analysis**):
  represented by dots and showing the calculated growth potential from the three replicates according to;
  Median difference (EURL, 2014), ●
  Mean difference (EURL, 2021), ●
  Maximum increase (Vermeulen et al., 2011), ○

- M (**Models**):
  represented by triangles and showing the calculated growth potential from models;
  Square root model (EURL, 2014), ▲
  Square root + lag time model (EURL, 2021), ▲
  Combase growth model, △
  DMRI dynamic safety model, ▲

Figure 5. Comparison of growth potential observations in minced pork with the calculated growth potential values from the analyzed data and models.