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META-REGRESSION MODELS DESCRIBING THE EFFECTS OF ADDED LACTIC ACID BACTERIA ON PATHOGEN INACTIVATION IN MILK AND CHEESE

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KEYWORDS

Biopreservation, starter culture, antimicrobial activity, mixed-effects linear model, meta-analysis.

ABSTRACT

Biopreservation methods based on the use of lactic acid bacteria (LAB) have been proposed as hurdles to increase the microbiological safety of many products, including fermented milk and cheese. For that reason, the objective of this research was: (i) to collect all available literature on *Bacillus cereus*, *Clostridium perfringens*, *Listeria monocytogenes*, *Listeria innocua*, *Staphylococcus aureus* and *Escherichia coli* inactivation in milk and cheese containing LAB; and (ii) to harmonise the retrieved data by constructing two separate meta-regression models that summarise LAB effectiveness.

After systematic review, 426 observations on log reduction data were extracted from twenty studies. The results suggest that exposure time, antimicrobial and pathogen's inoculum concentrations and biopreservative method of application are related to LAB antimicrobial effectiveness. Furthermore, interaction between bacterium and exposure time was found, revealing the distinct LAB inhibitory effect on different pathogens for the same exposure time.

One model also showed that, generally, higher microbial reduction can be achieved when LAB are added to milk, while application into cheese surface or mixture tend to present lower antimicrobial effect, even if still adequate for pathogen control. Globally, the results of these meta-regression models highlight the opportunity for increased microbial safety of dairy fermented products by adding functional starter cultures.

INTRODUCTION

Throughout history, lactic acid bacteria (LAB) have been linked with food fermentations as they promote a pH decrease that inhibits the growth of spoilage agents. Nonetheless, in the last years, several studies have reported the selection of LAB strains as functional starter cultures to be incorporated in a variety of foods products due to their potentiality as sources of antimicrobial metabolites, as a way to improve food microbiological quality and safety. The most recently researched antimicrobial metabolites produced by

LAB have been bacteriocins, and other main mechanisms of microbial inhibition by LAB include competition for substrates, production of antimicrobial substances (other than bacteriocins) and production of organic acids and other non-proteinaceous compounds (Tulini 2014).

Bacteriocinogenic LAB used in starter cultures are hurdles that can be used to replace other preservatives (chemicals, for instance) for pathogen inactivation, thus justifying the current large interest in this alternative solution by industries that are willing to go clean-label, and consumers that developed a high awareness towards the ingested foods, aiming to consume minimally manipulated, additive-free products.

Bacillus cereus, *Clostridium perfringens*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Escherichia coli* are well known common agents causing foodborne illnesses that can be found in numerous food matrices. In particular, these pathogens can be found in milk and in different types of dairy products, such as fermented milk and cheese (Iannetti et al. 2016; Jackson et al. 2018; Rosengren et al. 2010; Kousta et al. 2010; Cremonesi et al. 2007; Almeida et al. 2007; Tekinşen and Özdemir 2006; Morandi et al. 2019; Sadek et al. 2017).

Available literature has made clear the importance of improving the safety of dairy fermented foods to reduce pathogen growth, and, in this sense, there are several opportunities to incorporate selected LAB in such products. With many studies available describing the effect of this biopreservation method against several bacteria, it was considered that a meta-analysis of the published results will help evaluate added LAB usefulness to control foodborne pathogens in dairy products.

In this meta-regression study, the population is defined as milk or cheese with added LAB, and the measured outcome is the mean log reduction of the aforementioned pathogens. The results of this research intended to help improve the microbiological safety of cheeses and other dairy fermented products, and to optimise this hurdle technology by delivering an insight on the effects of LAB when added to milk or cheese, and on other factors and possible interactions that may influence microbial inactivation.

METHODOLOGY

Data Collection

Electronic, systematic literature search was carried out in Scopus, PubMed and Web of Science databases to find original and review articles, published since 2000, summarising biopreservation methods currently tested and/or applied in cheese-making and their efficiencies against *B. cereus* (BC), *C. perfringens* (CP), *L. monocytogenes* (LM), *L. innocua* (LI), *S. aureus* (SA) and *E. coli* (EC). The search aimed to find quality studies validated by the scientific community.

The bibliographic searches were performed by properly applying the AND and OR logical connectors to combine terms related to biopreservation and terms referring to biopreservatives characteristics and capacities in the selected products, as follows: (“starter culture” OR starter OR preservative OR extract OR bio-preservati* OR biopreservati* OR “lactic acid bacteria” OR “essential oil”) AND (antimicrobial OR inhibitory OR natural OR plant OR functional) AND (activity OR capacity OR propert* OR effect*) AND (cheese OR “fermented milk”). Grey literature was not considered to avoid data validity concerns and data duplication, as high-quality theses and reports are likely to be also published in peer-reviewed journals. Other meta-analysis studies and systematic reviews were also excluded as meta-analyses must be conducted with primary studies only. However, the individual studies referred to in the meta-analyses/systematic reviews retrieved were collected if not located by the bibliographic search.

After assessing the information from all publications, twenty studies published from 2000 until February 2019 were considered appropriate for inclusion, from which 426 observations on log inactivation data were retrieved (Achemchem et al. 2006; Callon et al. 2016; Cárdenas et al. 2016; Cavicchioli et al. 2017; Charlier et al. 2008; Cosentino et al. 2012; Costa et al. 2019; Langa et al. 2018; Mehdizadeh et al. 2018; Morandi et al. 2019; Oldak et al. 2017; Perin et al. 2013; Radovanovic and Katic, 2009; Rodríguez et al. 2005; Rolim et al. 2015; Røssland et al. 2003; Sadek et al., 2017; Sahraoui et al. 2015; Tetili et al. 2017; Vandera et al. 2017). The criteria for inclusion were: (i) Absence of competitive bacteria other than the added LAB (nevertheless, presence of natural microflora was accepted); (ii) Absence of chemical preservatives; and (iii) Reduction data greater than 0 log CFU/g or CFU/mL. When the information was presented only in graphical format, WebPlotDigitizer Version 3.8 was used to obtain the data points.

From each study, the following information was extracted: study ID, antimicrobial specific name, pathogen mean log reduction, storage temperature, exposure time, antimicrobial and pathogen inoculum concentrations and antimicrobial application (i.e., milk, cheese mixture, or cheese surface). The application type “milk” refers to the direct addition of the antimicrobial agent in bulk milk before curding; while the application type “cheese surface” describes the practice of smearing the cheese surface with the tested antimicrobial.

The application type “cheese mixture” was created to accommodate those challenge studies whose experimental methodology consisted of shredding the cheese, inoculating it with the pathogen, and adding the antimicrobial. Thus, “cheese mixture” does not reflect a real mode of application of antimicrobials in the cheese manufacturing process context, but an experimental protocol for challenge studies that researchers have probably devised for being handy although not realistic. To simplify, the levels of antimicrobial application “cheese mixture” and “cheese surface” are also referred to as “mixture” and “surface,” respectively.

To obtain precise estimates of the antimicrobial effect on pathogen inactivation and reflect quality of research design, different weights were assigned to each primary study (j) according to the sample size (n) used along the experiment to evaluate microbial inactivation. When a source did not present the number of replicates sampled to calculate the pathogen reduction, n=3 was assigned, since this was the modal value in the database.

Meta-Regression Models

Mixed-effects linear models with weights were separately adjusted to the milk and cheese data sets to evaluate the antimicrobial effect of added LAB on the square-root of log reduction (\sqrt{R}) of several foodborne pathogens. This transformation of the response variable was realised in order to approximate data normality. Variables defined for data analyses encompassed application type (App), exposure time (t), antimicrobial concentration (C) and inoculum concentration (Inoc). Due to lack of or uneven data, not all levels could be evaluated in the meta-regressions. The two meta-regression models carried out are described below:

$$\sqrt{R}_{ik} = \beta_{0i} + \beta_1 C + (\beta_2 + \beta_{3k}) \times \sqrt{t} + \varepsilon_{ik} \quad (1)$$

$$\sqrt{R}_{ikm} = \beta_{0i} + \beta_{1m} \text{App}_m + \beta_2 \text{Inoc} + (\beta_3 + \beta_{4k}) \times \sqrt{t} + \varepsilon_{ikm} \quad (2)$$

Equations (1) and (2) describe the meta-regression models used to evaluate the antimicrobial effect of LAB on pathogens in milk and cheese, respectively. The model in Equation (1) was used to evaluate the inhibitory effect of LAB on BC, LM and SA in milk, while the model in Equation (2) evaluates the LAB antimicrobial effect on BC, CP, LI and EC. The two equations contain different terms as some moderators were not introduced because they were confounded with other variables, not significant or due to lack of data. The variable exposure time was square-root transformed to reduce heteroscedasticity.

In Equation (1), β_{0i} is an intercept and β_1 represents the effect of a one log increase in antimicrobial concentration on the square-root of log mean reduction. In Equation (2), β_{0i} is again an intercept, β_{1m} is the set of fixed effects of the m types of application (a class variable consisting of the levels mixture, milk and surface), and β_2 is the effect of a one log increase in inoculum concentration on the square-root of log mean reduction. The parameters β_2 from Equation (1) and β_3 from Equation (2) represent the effect of exposure time on the mean log reduction. The parameters β_{3k} and β_{4k} from

Equation (1) and (2), respectively, allow the square-root of exposure time slopes to be different for distinct microorganisms.

The error terms ϵ_{ik} and ϵ_{ikm} from Equation (1) and (2), respectively, account for the variability between different antimicrobials i , pathogens k and antimicrobial applications m . The remaining unexplained variability was extracted by placing random effects due to antimicrobial type in the intercept (u_i), which was assumed to have a normal distribution with mean zero and variance s_u^2 .

Statistical Analysis

Model parameters, as affected by moderators, were calculated from the fitted meta-regressions, and the significance of moderators was evaluated by analysis of variance ($\alpha=0.05$). Additionally, to evaluate the variability between LAB single strains or cocktail of strains used, heterogeneity analysis was conducted. Moreover, assessment of the goodness-of-fit and histogram of Pearson's residuals (estimates of experimental error calculated from the difference between the observed values and the predicted values) was performed to verify the robustness of the model. All meta-regression models described were fitted using the *lme* function from the *nlme* package implemented in R Studio Version 1.2.5033.

RESULTS AND DISCUSSION

Antimicrobial Effect of LAB on BC, LM and SA in Milk

The results of the analysis of variance of the meta-regression model adjusted to evaluate the antimicrobial effect of LAB on BC, LM and SA inactivation in milk are presented in Table 1.

Table 1: Test of Fixed Effects of the Meta-Regression Models Predicting the Square-Root of Log Reduction (Log CFU/ml) of BC, LM and SA in Milk with Incorporated LAB as a Function of Moderating Variables

| Fixed effects | F-value | Pr > F |
|--|---------|--------|
| Antimicrobial concentration | 13.26 | 0.001 |
| $\sqrt{\text{Exposure time}}$ | 149.7 | <.0001 |
| $\sqrt{\text{Exposure time}} * \text{Bacterium}$ | 17.92 | <.0001 |

The significance of the terms antimicrobial concentration ($p=0.001$) and $\sqrt{\text{exposure time}}$ ($p<.0001$) reveal that such variables have an impact on the microbial reduction promoted by this type of biopreservative for all pathogens under study. The significant interaction term " $\sqrt{\text{exposure time}} * \text{bacterium}$ " ($p<.0001$) indicates that exposure time not only influences microbial inactivation by itself ($p<.0001$), but that it is also dependent on the bacterium. This means that, for a certain antimicrobial, distinct inhibitory effects may be achieved when implementing the same exposure time, depending on the pathogen that is being targeted. The results of the model built for the antimicrobial effects of added LAB on BC, LM and SA growth are presented in Table 2.

Table 2: Parameter Estimates of the Meta-Regression Model Predicting the Square-Root of Log Reduction (Log CFU/ml) of BC, LM and SA in Milk With Incorporated LAB as a Function of Moderating Variables

| Parameters | Mean | SE | Pr > t |
|---|--------|-------|---------|
| Predictors of $\sqrt{R_{ij}}$ | | | |
| β_{0i} (intercept) | -0.504 | 0.441 | 0.255 |
| β_1 (antimicrobial conc.) | 0.188 | 0.066 | 0.005 |
| β_2 ($\sqrt{\text{exposure time}}$) | 0.942 | 0.119 | 0.000 |
| β_{3k} ($\sqrt{\text{exposure time}} * \text{bacterium}$) | | | |
| <i>B. cereus</i> | 0 | - | - |
| <i>L. monocytogenes</i> | -0.346 | 0.123 | 0.006 |
| <i>S. aureus</i> | -0.647 | 0.129 | 0.000 |
| Variances | | | |
| s_u | 0.630 | | |
| s (residual) | 0.231 | | |

The significant positive mean values of β_1 and β_2 (0.188 and 0.942, respectively) indicate a tendency for greater microbial reduction as the antimicrobial concentration applied and the exposure time increase; however, while antimicrobial concentration and mean log reduction reveal a linear relation, exposure time and mean log reduction are not linearly correlated. As shown by the results of the analysis of variance, microbial inactivation is time and bacterium dependent. In fact, based on the results of Table 2, it is possible to observe that distinct pathogens suffer different log reductions when the same antimicrobial is applied over the same exposure time, as demonstrated by the various mean values of the β_{3k} parameter.

For better understanding of the meta-regression results, it is important to note that the estimate for BC is considered the "base value" for microbial reduction, with mean zero, and the estimates for other microorganisms reflect deviations from that base value, with negative values below the microbial reduction base and positive values above the base. In this sense, the results show that BC suffers the highest microbial reduction when challenged with LAB, followed by LM (-0.364), while SA appears to be the most resistant pathogen to the presence of such biopreservative (-0.647). In other words, when added to milk, LAB promote faster inhibitory effect for BC than for LM or even SA.

For this meta-regression, the heterogeneity analysis conducted revealed that 11.64% of the between-antimicrobial variability could be explained by the moderators introduced in the model, meaning that, for each antimicrobial, the results differed from study to study due to distinct antimicrobial concentrations applied, exposure time used and targeted bacteria. However, there is still some residual variability to be explained that this model cannot account for. Possible sources of variation are type of milk (raw versus pasteurised), temperature of fermentation/ripening and application of selected single LAB strains versus the use of cocktails of selected LAB strains. In order to evaluate the quality of the model produced, the goodness-of-fit was

assessed and the histogram of Pearson's residuals was built, as shown in Figure 1 and Figure 2, respectively.

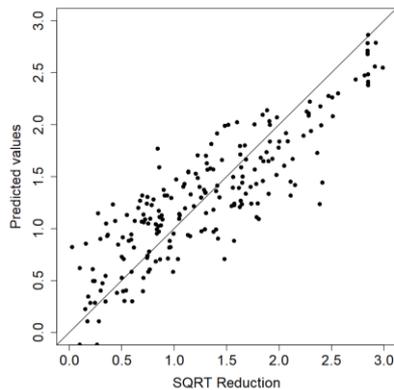


Figure 1: Goodness-of-Fit of the Meta-Regression Model Predicting the Square-Root of Log Reduction (Log CFU/ml) of BC, LM and SA in Milk With Incorporated LAB

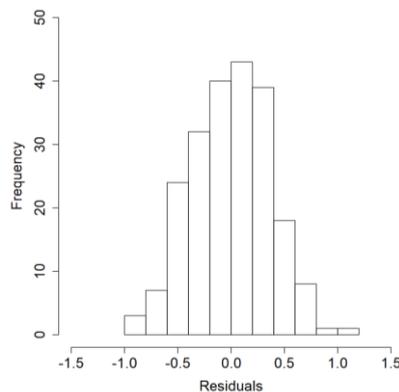


Figure 2: Histogram of Pearson's Residuals of the Meta-Regression Model Predicting the Square-Root of Log Reduction (Log CFU/ml) of BC, LM and SA in Milk With Incorporated LAB

The goodness-of-fit shown in Figure 1 shows a correlation value acceptable for a meta-analysis study ($R=0.885$). In terms of the residuals, these can be thought as elements of variation unexplained by the fitted model, to which the same general assumptions used for errors apply: it is expected that residuals are roughly normal and approximately independently distributed, with a mean of 0 and constant variance. The histogram is a fast, graphical method to evaluate residuals, and as seen from Figure 2, the residuals of this meta-regression model are symmetrically distributed around zero, as desired. According to the goodness-of-fit and the histogram obtained, it can be stated that the model produced is robust.

Antimicrobial Effect of LAB on BC, CP, EC and LI in Cheese

The results of the analysis of variance of the meta-regression model built to evaluate the inhibitory effect of LAB when added to cheese on BC, CP, EC and LI reduction are shown in Table 3.

Table 3: Test of Fixed Effects of the Meta-Regression Models Predicting the Square-Root of Log Reduction (Log CFU/g or ml) of BC, CP, EC and LI in Cheese with Incorporated LAB as a Function of Moderating Variables

| Fixed effects | F-value | Pr > F |
|--|---------|--------|
| Application type | 13.46 | <.0001 |
| Inoculum concentration | 45.23 | <.0001 |
| $\sqrt{\text{Exposure time}}$ | 339.6 | <.0001 |
| $\sqrt{\text{Exposure time}} * \text{Bacterium}$ | 81.96 | <.0001 |

All terms included in the model revealed to be significant ($p<.0001$). More specifically, this means that the application method of the antimicrobial and the pathogen contamination level have an impact on the microbial inactivation achieved from the incorporation of LAB in cheese, as well as the exposure time to which the cheese is subjected to the biopreservatives. As in the previous model for the incorporation of LAB in milk, the term " $\sqrt{\text{exposure time}} * \text{bacterium}$ " was also included since it was found to be significant ($p<.0001$), indicating that each pathogen would have its own rate of inactivation as affected by added LAB.

Table 4 presents the estimates results of the meta-regression produced regarding the antimicrobial effects of added LAB on BC, CP, EC and LI growth in cheese. The β_{1m} parameter provides further insight regarding the differences in inhibitory capacity of LAB when added to cheese by distinct application methods, as previously observed through the result of the analysis of variance, where application type was found to be a significant term ($p<.0001$). In this case, the "base value" is the application of LAB in cheese mixture, and it can be stated that incorporation in cheese surface ($p=0.246$) leads to the same inhibitory effect than cheese mixture. On the other hand, application in milk is significantly different from cheese mixture and surface, and provides the highest antimicrobial effect of LAB on pathogens, as indicated by the positive mean estimate value (0.745).

The parameter describing the effect of inoculum concentration, β_2 , suggests an inverse relationship between this variable and microbial log reduction (-0.244). This could be explained by the fact that an increase in cell numbers boosts the probability of growth, even under suboptimal conditions, thus limiting antimicrobial inactivation (Koutsoumanis and Sofos, 2005). However, some studies have also demonstrated that inoculum concentration should have no effect on the growth kinetics (Bidlas et al. 2008; Buchanan et al. 1993). For this reason, more research towards this topic is needed in order to better understand how the inoculum level could have an impact on microbial reductions.

Table 4: Parameter Estimates of the Meta-Regression Model Predicting the Square-Root of Log Reduction (Log CFU/g or ml) of BC, CP, EC and LI in Cheese With Incorporated LAB as a Function of Moderating Variables

| Parameters | Mean | SE | Pr > t |
|--|--------|-------|---------|
| Predictors of $\sqrt{R_{ikm}}$ | | | |
| β_{0ik} (intercept) | 1.550 | 0.302 | 0.000 |
| β_{1m} (application type) | | | |
| Mixture | 0 | - | - |
| Milk | 0.745 | 0.181 | 0.000 |
| Surface | -0.272 | 0.227 | 0.246 |
| β_2 (inoculum conc.) | -0.244 | 0.058 | 0.000 |
| β_3 ($\sqrt{\text{exposure time}}$) | 0.325 | 0.015 | 0.000 |
| β_{4k} ($\sqrt{\text{exposure time}}$ * bacterium) | | | |
| <i>B. cereus</i> | 0 | - | - |
| <i>C. perfringens</i> | 0.001 | 0.019 | 0.940 |
| <i>E. coli</i> | -0.348 | 0.036 | 0.000 |
| <i>L. innocua</i> | -0.319 | 0.023 | 0.000 |
| Variiances | | | |
| S_u | 0.217 | | |
| s (residual) | 0.186 | | |

Similar to the results of the previously presented meta-regression model, describing the effects of LAB on BC, LM and SA in milk, the term $\sqrt{\text{exposure time}}$ was found to be significant ($p=0.0001$), meaning that longer exposure times of the cheese to the antimicrobial lead to increased microbial reduction. However, as this is not a linear correlation, it also suggests that the inhibitory effect tends to stabilize around a certain value for increased exposure times. Lastly, the β_{4k} parameter, describing the interaction between $\sqrt{\text{exposure time}}$ and bacterium, reveals that, for the same exposure time used, the inhibitory effect of LAB on BC and CP growth will be virtually the same ($p=0.940$). Mean levels of reduction for EC and LI are also not significantly different from one another, but inactivation appears to be more challenging for these two pathogens than for BC and CP, as revealed by the negative mean values -0.348 and -0.319, respectively.

Even though literature has suggested a broad spectrum of inhibition promoted by LAB against both Gram-positive and Gram-negative bacteria (Šušković et al. 2010), as the results of this meta-regression also suggest, the bacteriocinogenic LAB may have a different inhibitory behaviour depending on the pathogenic microorganism. If the main antimicrobial mechanism of the selected LAB is the production of bacteriocins, this could be a drawback, as these compounds mainly inhibit closely related organisms, which could mean that bacteriocin-producing LAB strains may not be able to inhibit Gram-negative pathogens, but only Gram-positive ones (Mokoena, 2017). However, this meta-regression model does not provide insight on such matter as there is not enough data to draw conclusions. Nevertheless, it is important to retain this information and possibly promote further studies of this issue to optimise, if necessary, the use of this hurdle technology according to the pathogen of concern.

Heterogeneity analysis of this meta-regression model was carried after assessment of the parameters estimates, and revealed that 78.87% of the between-LAB variability could be explained by the moderators included in the model. The percentage of variability explained by this model is considerably higher than the one explained by the previous model, which is mainly due to the inclusion of a larger number of moderators that are able to absorb variability. Finally, the quality of the model produced was assessed by evaluating the goodness-of-fit and the histogram of Pearson's residuals, shown in Figure 3 and Figure 4, respectively.

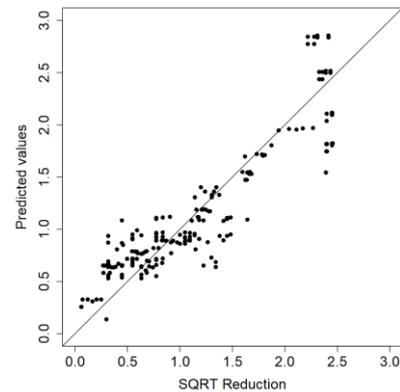


Figure 3: Goodness-of-Fit of the Meta-Regression Model Predicting the Square-Root of Log Reduction (Log CFU/g or ml) of BC, CP, EC and LI in Cheese With Incorporated LAB

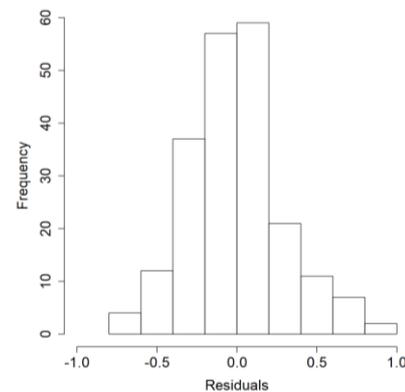


Figure 4: Histogram of Pearson's Residuals of the Meta-Regression Model Predicting the Square-Root of Log Reduction (Log CFU/g or ml) of BC, CP, EC and LI in Cheese With Incorporated LAB

In this meta-regression model, the goodness-of-fit was found to be $R=0.916$, a considerably high correlation value, and Figure 4 shows the residuals to be symmetrically distributed around zero. The plots illustrated by both figures support the robustness of the model produced.

CONCLUSION

Data collected from literature was valuable to build meta-analytical regression models that summarise the antimicrobial effects of added LAB on pathogen growth in both milk and cheese. It was also possible to obtain insight on the many variables involved in the effectiveness of such biopreservative, in particular the antimicrobial concentration

used, the exposure time selected and method of application of the biopreservative (cheese mixture or surface, or added to milk). Both models produced suggested the significant correlation between exposure time and microbial reduction, as well as the interaction between this term and bacterium, thus revealing the distinct results that can be obtained by a specific antimicrobial depending on the targeted microorganism, for the same exposure time.

The results of the meta-regression model for the antimicrobial effect of LAB on *B. cereus*, *L. monocytogenes* and *S. aureus* in milk revealed that, in general, this hurdle technology will have a greater impact on *B. cereus* growth than on the other two pathogens, for the same exposure time, with *S. aureus* appearing to be the most resistant pathogen to LAB inhibitory action. The second meta-regression model tested, built to evaluate LAB antimicrobial effect on *B. cereus*, *C. perfringens*, *E. coli* and *L. innocua*, showed the significant impact of application type on microbial reduction. Furthermore, it revealed that antimicrobial application to cheese mixture or surface are expected to lead to similar inactivation, while application in milk, at an earlier stage of the cheese manufacturing process, should be able to promote higher microbial reduction. In this study, no difference was observed with regards to the antimicrobial spectrum of action of LAB, as Gram-positive and Gram-negative bacteria did not present significantly different results.

Overall, this research shows that meta-regression modelling may help obtain a greater understanding on the main variables influencing microbial reduction when biopreservatives such as functional starters are to be included in fermented dairy products. This information can be useful for the experimental design of challenge studies and for the optimisation of the use of lactic acid bacteria as a biopreservation technology for pathogen control in foods to ensure microbial quality and safety.

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