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MODELLING THE GROWTH KINETIC OF SPOILAGE MICROORGANISMS IN A PACKAGED COW'S RICOTTA PROCESSED WITH HIGH PRESSURE

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ABSTRACT
Today consumers demand fresh foods without additives, preservatives and health risks: that is why non-thermal food preservation methods are receiving more interest, among them High Pressure Processing is able to avoid thermal degradation of food components, extend their shelf life and preserve colour, flavour and nutritional value. HPP is often used on dairy products because of its impact on physicochemical and sensory characteristics, its ability to improve their structure and texture and inactivate some microorganisms.
The aim of this work is to evaluate the effect of HPP on a packaged ricotta rich in Conjugated Linoleic Acid (CLA) and Omega-3, resulting from cows fed with linseed in the Parmigiano Reggiano area, and processed with a hydrostatic pressure of 600 MPa for 5 minutes. The ultimate goal is to find a mathematical model able to show the treatment's effect on spoilage microorganisms that grow spontaneously in this product during a month of refrigerated storage.

Keywords: High Pressure Processing, Modelling, Ricotta, shelf life, Milk

1. INTRODUCTION

In the last years consumers demand fresh food with high sensorial and nutritional quality, but additive free, mildly processed, and especially safe from pathogenic and spoilage microorganisms. Therefore, food companies are challenging to develop food products with consumer-desired characteristics, using advanced thermal based technologies such as aseptic processing, microwave, ohmic and radio-frequency heating (Balasubramaniam et al. 2015). Moreover, the adoption of cleaner condition during the packaging phase could help to increase the shelf life of products (Bertolini et al. 2016). The major advantage of thermal treatment is the inactivation of pathogenic and spoilage microorganisms, with the consequent increasing of food shelf life, but it affects product's quality as nutritional value and color, disrupts vitamins and changes food flavor (Mosna and Vignali 2016). Thus, non-thermal methods have been developed such as irradiation, pulsed electric field, UV and High Pressure Processing (HPP) that has been recognized as one of the more relevant and frequently applied alternative techniques to conventional thermal processing.
The non-thermal pasteurization effect of high pressure on foods has been known since the 19th century: in 1899 Hite used hydrostatic pressure up to 600 MPa as a tool to preserve milk, then in 1914 it was tested on vegetables and fruits, but the major revolution came in Japan by releasing the first high pressure processed product into market (Elamin et al. 2015).
Over the past 30 years many studies have been performed to understand advances of HPP and it has been successfully implemented in different type of food industries worldwide. Nowadays, it is used in Japan, United States and Europe for pasteurization of food products, and equipment installation are increasing in numbers: the most famous and important industries of HPP machines today are Avure Technologies and Hyperbaric.
The major type of industrial high-pressure operating system is the batch-one, in which the cycle is characterized by the loading, compression, holding, decompression and unloading phases. The equipment usually consists of a cylindrical pressure vessel and its closure, an intensifier pump to generate pressure and a material-handling system. The food product, previously sealed in flexible and water-resistant packaging (Lambert et al. 2000), is put in a cylindrical container, pushed and closed into the pressure vessel: a pressure medium, usually cold water, is pumped isostatically from its tank and once the desired pressure is reached, the pump is stopped by closing the inlet valves. The high pressure transmitted by water on foods is usually up to 400-600 MPa for 3 up to 30 minutes (Elamin et al. 2015).
A variety of liquid and solid foods can be treated with batch application, but since pasteurization treatments do not typically inactivate bacterial spores, it is important to maintain pressure-pasteurized products under refrigerated storage after the treatment. In fact, bacterial spores are more resistant to high hydrostatic pressure than vegetative cells: they can be eliminated with repeated cycling between high and low pressures, but the risk is only the partial elimination of the germinated spores, that can cause food poisoning. Instead, HPP successfully destroys vegetative microorganisms thanks to irreversible change to the membranes and the

disruption of nucleic acids and ribosomes. The consequent permeabilistation of the membranes and the leakage of the content involve the cell's death (Datta and Deeth 1999).
Thanks to the destruction of spoilage microorganisms, HPP extends the shelf life of some products up to 10 times. The consequence is that additives or preservatives are not necessary and foods are more natural. Moreover, with the destruction of pathogens as *Listeria*, *Staphylococcus*, *Salmonella*, *Escherichia coli*, the technology assures food safety and exportation in worldwide markets. Today different markets in the world sell a wide range of products treated with HPP: juices, beverages, vegetable, fruit, meat, seafood products, ready to eat meals, soups, sauces, infant foods and dairy products. In 2014 HPP food production exceeded 500 million kg (Elamin et al. 2015).
In general, the complexity of food and the wide variety of phenomena that occur under pressure make difficult to predict effects of HPP on foods. For these reasons, HPP conditions must be evaluated in each specific product. Many researchers studied HPP effects on dairy products: the treatment seems able to improve their structure, homogenizing and softening ricottas and cheeses. Moreover, it increases lightness and conserve food colour for a long time (Calzada et al. 2015). Also a great amount of data exist on the effects of the treatment on chemical and microbiological characteristics of dairy products (Datta and Deeth 1999; Devi et al. 2013; Okpala et al. 2009; Pega et al. 2018; Ribeiro et al. 2018; Wibowo et al. 2019).
However, the largest part of researches consists in inoculating some microorganisms in foods and evaluating their survival after the treatment at different pressures and times, in order to estimate the destructive power of HPP (Serment-Moreno et al. 2017; Mussa et al. 1999; Serment-Moreno et al. 2015; Chen and Hoover 2003). Instead, there are very few studies on the spontaneous growth of microorganisms in foods treated with High Pressure Processing (Giacometti et al. 2016).
In the last years, in the Italian Parmigiano Reggiano area, some cows are fed with linseed rich in CLA and Omega 3: the nutritional value of their food is transmitted in their milk, and consequently in cheeses and ricottas produced from it. Since the advantages of HPP seem to be very promising on cheeses and dairy products, in this work the treatment was used on ricotta in order to maintain its nutritional value (Gutiérrez 2016) and simultaneously improve its shelf life. Ricottas processed with high pressure were stored in fridge with untreated ricottas and observed for 30 days: every 10 days some ricottas were analysed in order to study the spontaneous microbial growth. Finally, the influence of the treatment has been studied using primary models to describe the growth of spoilage microorganisms.

2. MATERIAL AND METHODS

Natural ricotta has a really short shelf life because of the pH above 6.0 and water activity between 0.974-0.991 that make this product an excellent growth medium for a wide range of microorganisms (Pala et al. 2016). Ricotta is created from the whey obtained from the milk: microbes may originate on the farm from the milking equipment, environment or employees. Even if the production process reach elevated temperature that kills psychrophilic and mesophilic bacteria, the heat-tolerant species could survive and cause deterioration (Rawat 2015). Moreover, ricotta is exposed to microbial secondary contamination due to equipment and processing environment if the hygienic rules are not well respected in the processing plant.
Spoiled ricotta may be safe to eat if there are no pathogens or toxins, but it is characterized by undesirable or unacceptable sensory modifications for human consumption as off-odours or flavours and visible changes in colour and texture. Instead, it may do not look bad even if it is severely infected by pathogenic bacteria, and for this reason bacterial contamination is more dangerous.
The main spoilage and pathogenic microorganism that can be found in ricotta are *Enterobacteriacea*, *Listeria monocytogenes*, *Pseudomonas* spp., *Bacillus cereus*, yeasts, moulds and lactic acid bacteria. There are many parameters that establish the admissible concentration of microorganisms in ricotta suitable for consumers and many standards methods to conduce the microbiological analyses. Firstly, in order to ensure the non-alteration of the food, the concentration of total aerobic mesophilic bacteria have to be less than 10^8CFU/g. The optimum count in ricotta and soft cheese is $<10^6$ CFU/g. Instead, if the number of bacteria is between 10^6 and 10^7 CFU/g it's microbiologically acceptable, but it denotes that the hygiene of cheese production must be improved. Thus, if the concentration is $>10^7$ CFU/g it is unacceptable for human consumption, and it means that the process must be rechecked from a microbiological point of view.
On the other hand, a satisfactory concentration of yeasts and moulds is $<10^2$ CFU/g (FCD 2009), but it is also acceptable between 10^2 and 10^3 CFU/g.
The presence of *Pseudomonas* spp. (optimum if $<10^6$ CFU/g) and *Bacillus cereus* (optimum if $<10^2$ CFU/g) has to be evaluated during or at the end of the productive process, while during the food shelf life is advisable to study the presence of *Listeria monocytogenes* (must be absent or <100 CFU/g) and *Salmonella* spp. (absent).

2.1 Packaging and HP treatment

Depending on the target markets, ricotta is packaged in different ways: it can be wrapped in food paper for the gourmet food store, while for the large-scale retails it is usually packaged in vacuum or in Modified Atmosphere Packaging (MAP) in order to extend its shelf life. On the other hand, High Pressure Processing seems able to increase shelf life of dairy products up to 4 or 5 weeks (Pega et al. 2018; Giacometti et al. 2016).
In previous researches (Ugolotti and Vignali 2018), ricottas packaged in MAP were suitable for 10 days, but in this work the desired shelf life was at least twice as much as reached before: for that reason the HPP was used. Using the EasyFrom machine by ILPRA (ILPRA

EasyForm Machine) 16 thermoformed trays containing 500g of ricotta were created. Trays were composed by PET/EVOH/PE, with a depth of 20mm and a little head space containing not MAP but atmospheric air.
The half part of the trays were treated at 600 MPa for 5 minutes in Avure Technologies HP machine (HPP Machine QFP350L). Then, the 8 untreated and 8 HPP trays were refrigerated at 4°C for a month.

2.2 Microbiological analyses

Every 10 days (T0=0 days, T1=10 days, T2=20 days, T3=30 days), 2 treated and 2 untreated trays were analysed in order to observe the concentration of spoilage microorganisms in ricottas, despite the conservation in the fridge was surely able to slow down their growth.

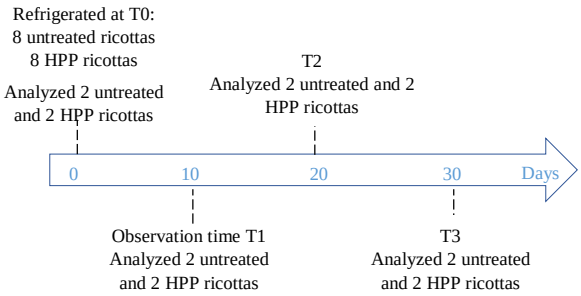

Figure 1: Planned Observation Times

The main object of study was the invasion by microorganisms such as moulds, yeasts and bacteria that cause spoilage in ricottas and were responsible for sensory changes which make ricottas unacceptable for consumers' consumption. For that reason, the presence of some pathogenic bacteria as *Listeria monocytogenes*, *Bacillus cereus* and *Salmonella* spp. was not investigated: moreover, all the previous analyses done on this type of ricottas testified that their concentration was equal to zero.
Therefore, analyses searched for lactic acid bacteria (LAB) that are a group of gram-positive bacteria responsible of spoilage under low oxygen, low temperature and acidic conditions: their undesirable changes in milk and derivatives are off-flavours, gas formation in cheeses (blowing) and acidification (Rawat 2015). Moreover, the presence of negative nuclease cocci and Enterobacteria was investigated because they could be an index of non-hygienic production process. Finally, the total bacteria count was analyzed taking account of all types of bacteria that grew up in ricottas.
Analyses were conducted at illustrated times (Figure 1) with the same method: approximately 20g of ricotta in each tray were diluted with 9 parts of citrate buffer (trisodium citrate 20g/L) and homogenized with Stomacher for 120 seconds. Then, the suspension was used for serial dilutions in physiological solution (NaCl 9g/L) inoculated in Petri dishes in order to search for microbial groups.
Finally, Crison pH-meter was used to measure the pH of each samples in order to take account of possible acidifications that occurred in ricottas.

Table 1: Analyses parameters and culture medium

Culture medium	Microbial group	Parameters
Violet red Bile Glucose Agar (VRBGA)	Enterobacteria	37°C, 48h
Plate count Agar (PCA)	Total bacterial count	30°C, 72h
Baird & Parker Agar	Negative nuclease cocci	37°C, 72h
Yeast Peptone Dextrose Agar (YPD)	Yeasts and molds	28°C, 5 days
MRS agar	Lactic acid bacteria	30°C, anaerobic, 5 days

2.3 Microbial growth curve

Microbial growth in foods could be compared to a batch culture in a growth medium, where nutrients are exhausted while metabolites are accumulated and could inhibit the cells growth. In this ecosystem, microbial growth is characterized by different phases:

1- *Lag phase*. Firstly, bacteria have to adapt to growth conditions: they start to synthetize enzymes and intermediate metabolites, but are not able to divide. The duration of this phase depends on the type of bacteria and environmental factors such as CO_2, temperature or pH.
2- *Log or exponential phase*. Cells start to reproduce into two daughter cells and their number increase exponentially. In the chart, a straight line represents this period: the slope is the specific growth rate of the organism, which is a measure of the number of division per cell per unit time.
3- *Stationary phase*. The growth and cell division cease due to exhaustion of nutrients, accumulation of toxic products or in general a growth-limiting factor. The phase is represented by a straight horizontal line. Growth rate and death rate are equal.
4- *Decline or death phase*. The total number of cells remains constant, but the number of viable cells gradually decreases due to lack of nutrients or other injurious conditions.

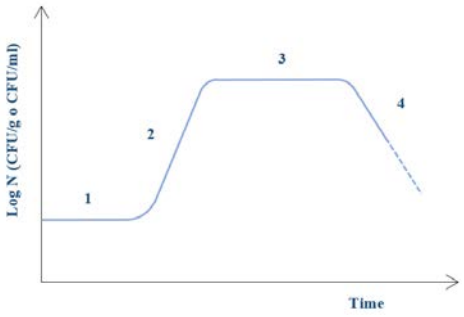

Figure 2: Microbial Growth Curve

2.4 Microbial growth models

In order to secure food safety, predictive microbiology creates models to predict microbial growth and inactivation in food products. In particular, primary models describes kinetics of growth, inactivation or

survival. Secondary models study the effects of environmental and process conditions of primary models parameters. Finally, mathematical expressions are collected into different software, called tertiary models, useful for researchers who want to study microbial growth in foods: ComBase Predictor and Pathogen Modeling Program are the most famous and used.
There is a large number of empirical sigmoidal equations of primary models: they are indispensable tools for the comprehension, prevision and control of phenomena caused by the growth of microorganisms, as food deterioration, pathogenic growth and metabolite production. The modelling purpose is the individuation of a mathematical expression able to predict the growth in every instant of time and estimate the most important parameters in every growth curve, as the lag phase duration (λ) and the maximum specific growth rate (μ_{max}). In particular, in order to create a safe food characterized by a long shelf life, the growth inhibition of pathogenic and spoilage microorganisms is desired: it means to prolong λ and reduce μ_{max}.
The Gompertz primary model is a sigmodal curve where the lag phase is calculated from a geometrical interpretation: it was often used in order to describe the microbial multiplication until the 90s. However, in 1994 Baranyi and Roberts studied a new mathematical approach for a mechanical model for the lag phase (Baranyi and Roberts 1994). Their model is the most widely used, because it can apply various environmental factors appropriately and interpret the calculated parameters biologically. The Baranyi equation is the following:

$$y(t) = y_0 + \mu_{max}A(t) - \ln(1 + \frac{e^{\mu_{max}A(t)}+1}{e^{(y_{max}-y_0)}}) \quad (1)$$

$$A(t) = t + \frac{\ln(e^{-\mu_{max}t}+e^{-h_0}-e^{-t-h_0})}{\mu_{max}} \quad (2)$$

In the model, y_0 is the initial bacterial counts, while y_{max} is the final bacterial counts.
In the equation (2) h_0 is called "work to be done": it's the work needed by the cell for the adaptation and the duplication. It could be expressed also by α_0 parameter, that is the "initial physiological state": it's equal to zero if cells don't duplicate (infinite lag phase), or $\alpha_0 = 1$ if all cells reproduce immediately ($\lambda = 0$). The expression of these parameters is reported in the following equations:

$$h_0 = \lambda\mu_{max} = \ln\left(1 + \frac{1}{q_0}\right) = -ln\alpha_0 \quad (3)$$

$$\alpha_0 = e^{-\mu_{max}\lambda} \quad (4)$$

To model microbiological data during the observation period, ComBase Predictor is used. It is possible to use it online or with the DMfit program provided for Excel. The program reports the estimated equation parameters and the coefficient of determination (R^2) and Squared Error (SE), which indicate the statistical fit of the primary model.

3. RESULTS AND DISCUSSION

3.1 Visible changes during observation

At T0, the main visible effect of the treatment is syneresis that is the separation of the aqueous phase of ricotta, due to the pressure. At T1, untreated ricottas started to change color: they become light-rose and trays started to swell up. The Oxybaby instrument, able to measure the percentages of gas inside each tray, showed that in bulgy packaging the Oxygen was completely consumed and Carbon Dioxide was produced: it was a clear index of fermentation in untreated ricottas. Contrariwise, HPP ricottas remained white and without visible change for 30 days.

Figure 3: Rose untreated ricottas and bulgy trays

3.2 Microbiological analyses results

Results show that, at the beginning of the observation, pH is very similar between untreated and HPP trays, but after 10 days, untreated ricottas acidified. Instead, acidification in ricottas treated at 600 MPa for 5 minutes started only after 30 days in the fridge at 4°C. Certainly, the result demonstrates the ability of high pressure to slow down chemical changes and reactions in foods.

Table 2: pH results

Time	Untreated	HPP
T0	5.15	5.13
T1	4.66	5.09
T2	5.74	5.10
T3	4.50	4.72

Microbiological results show that at T1 in untreated ricottas some microorganisms as negative nuclease cocci, yeast, molds and lactic acid bacteria started to grow up: of course the last one were the main cause of the acidification, fermentation and swelling. Instead, microbial growth in HPP ricottas started to be significant only after 30 days of refrigerated storage. Enterobacteria didn't grow up at any time in any ricottas (<10 CFU/g).

Table 3: Total bacterial count results [CFU/g]

Time	Untreated	HPP
T0	2.000	2.500
T1	1.600.000	4.000
T2	272.000.000	240.000
T3	490.000.000	13.200.000

Table 4: Negative nuclease cocci results [CFU/g]

Time	Untreated	HPP
T0	<10	<10
T1	1.040	80
T2	300.000	240
T3	600.000	380

Table 5: Yeasts and molds results [CFU/g]

Time	Untreated	HPP
T0	<100	<100
T1	400	1.000
T2	3.500	800
T3	4.500	1.200

Table 6: Lactic acid bacteria results [CFU/g]

Time	Untreated	HPP
T0	<10	<10
T1	5.400.000	5.000
T2	45.000.000	43.000
T3	50.000.000	12.000

In microbiology, in order to calculate the magnitude of the change in cell number, a logarithmic scale is often used. In fact, taking the log value of a large number transforms it into a smaller one that is easier to work with. Therefore, the number of cells CFU/g were transformed in log: the charts show the different bacterial growth in treated and untreated ricottas during a month.

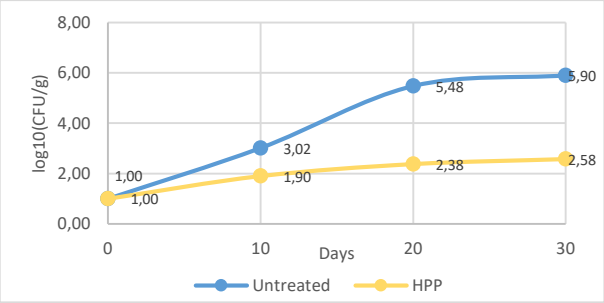

Figure 4: Nuclease negative cocci growth curve

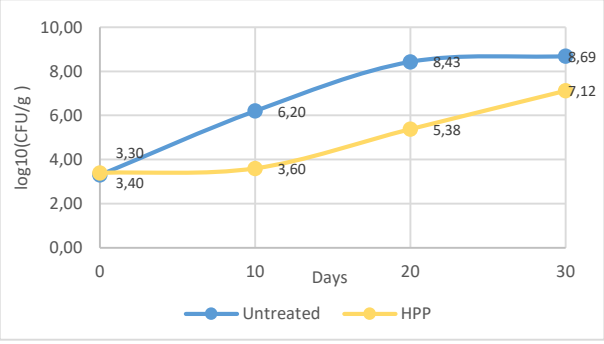

Figure 5: Total bacterial count growth curve

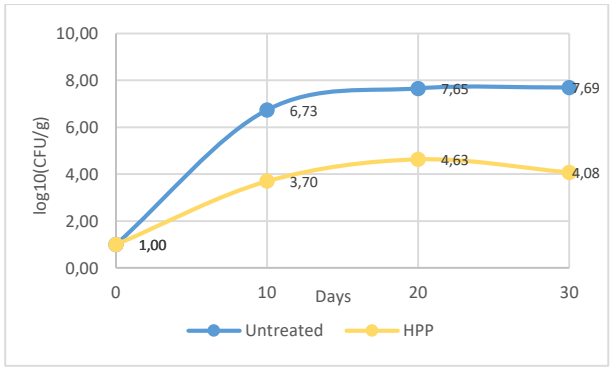

Figure 6: Lactic acid bacteria growth curve

Charts show that the microbial growth in HPP and untreated ricottas is very different. The untreated ricottas curves are similar to the exponential phase of the generic microbial growth illustrated in figure 2: it seems that, at T0, there are already the conditions of duplication of bacteria, who start to grow up quickly. Instead, the first section of HPP ricottas curves seems similar to the lag phase: cells need time to adapt to the new conditions of growth.

Especially, plots shows that the main difference between the two types of ricottas is the growth during time: in HPP ricottas is slower. In fact, using the following mathematical expression for each microorganisms at each observation time, it is possible to see that HPP inhibits bacterial growth from 1 to 3 log after 10 days, and up to 3 log after 20 days.

$$\log reduction = \log(HPP_{(Ti)}) - \log(untreated_{(T_i)}) \qquad (5)$$

In particular, looking at the curves, High Pressure seems able to prolong λ and reduce y_{max} and μ_{max} of acid lactic bacteria, negative nuclease cocci and total bacteria count. In other words, it means that HPP changes growth conditions in ricottas and does not allow cells to quickly reproduce. This is a very important result because prolonging the lag phase, the concentration of bacteria is lower and the food is safe for more time.

The content of acid lactic bacteria in untreated ricottas grows exponentially during time and after 20 days it's already greater than 10^7 CFU/g, showing an evident product's alteration. Instead, in HPP trays the number is lower, remaining less than 10^5 CFU/g also after 30 days, testifying the safety of ricottas. The total bacterial count at T2 is greater than 10^8 CFU/g in untreated ricottas, while in HPP ricottas is yet acceptable (10^5 CFU/g). Finally, yeast and molds concentration's limits are more respected in treated ricottas than in untreated ones.

Using ComBase Predictor online, the microbial growth curves were fitted with the most appropriate primary model among linear model, Baranyi and Roberts model and Biphasic model. On y-axis the concentration expressed in log CFU/g is reported, and on x-axis time is expressed in hours. In the chart, data points represent the analyses results, while the curves are the model created by the software.

In order to measure the adequacy of the model to describe data, the coefficient of determination R^2 was calculated: the higher is ($\cong 1$), the better is the adequacy.

$$R^2 = 1 - \frac{\Sigma(y-\hat{y})^2}{\Sigma(y-\bar{y})^2} \quad (6)$$

The microorganisms' growth in treated ricottas can be modelled with the equation of Baranyi and Roberts.
In particular, the content of acid lactic bacteria can be modelled with the equation of Baranyi and Roberts without the lag phase (R^2 = 0,942 and SE of fit = 0,389). The essential parameters that can describe the curve are the maximum growth rate μ_{max}, the initial value y_0 and the final value y_{max} (Table 7).

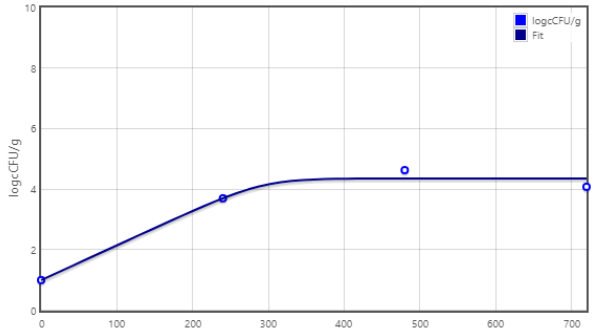

Figure 7: Lactic acid bacteria in HPP ricottas modelled with Baranyi and Roberts equation without lag phase

Total bacteria count can be represented by the same model but with no asymptote. R^2 value is very high (0,995) and SE of fit is 0,127. The maximum growth rate, the lag/shoulder parameter and the initial value are reported in table 7.

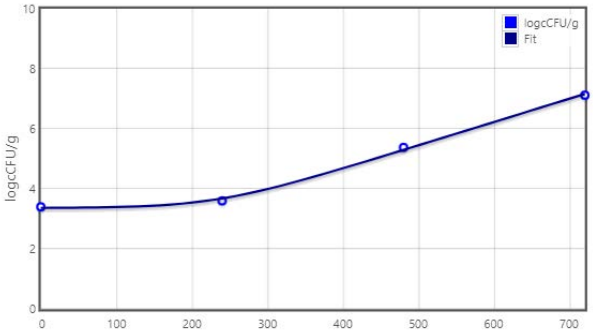

Figure 8: Total bacteria count in HPP ricottas modelled with Baranyi and Roberts equation without asymptote

Finally, also the content of negative nuclease cocci in HPP ricottas can be represented with Baranyi and Roberts model without lag phase, with R^2 = 0,968 and SE of fit = 0,126.

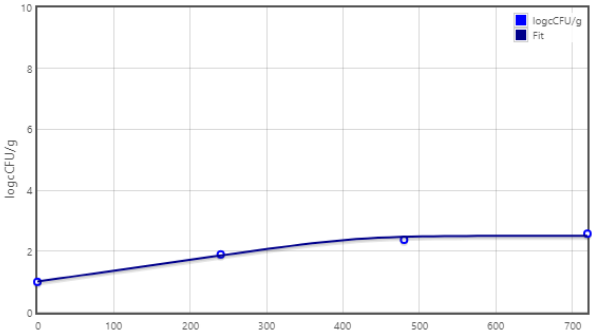

Figure 9: Negative nuclease cocci in HPP trays modelled with Baranyi and Roberts (no lag phase)

Table 7: Growth parameters in HPP ricottas

Baranyi-Roberts	y_0	y_{max}	μ_{max}	λ
Acid lactic bacteria	1,0000555 ±0,389	4,355 ±0,276	0,0115 ±0,00261	/
Total bacterial count	3,364 ±0,122	/	0,00779 ±0,000543	232,459 ±34,152
Nuclease negative cocci	1,00906 ±0,126	2,51 ±0,102	0,00358 ±0,000735	/

Finally, each microorganism's growth in untreated ricottas was modelled with ComBase using the Baranyi and Roberts Model without lag phase. Parameters are reported in the following table and charts are attached in appendix.

Table 8: Growth parameters in untreated ricottas

Baranyi-Roberts	y_0	μ_{max}	y_{max}	R^2	**SE**
Lactic Acid bacteria	1 ± 0,0281	0,0247 ±0,000208	7,67 ±0,0199	1	0,0281
Total bacterial count	3,305 ±0,042	0,012 ±0,000233	8,67 ±0,0375	1	0,0423
Nuclease negative cocci	0,921 ±0,266	0,00959 ±0,00116	5,826 ±0,281	0,985	0,277

Parameters' results confirm that the microbial growth in HPP ricottas is lower than in untreated ones. Considering the total bacteria count, the treatment prologs the lag phase, while in the others type of ricotta this phase is absent and cells start to reproduce immediately. Finally, as seen before, y_{max} of each microorganism is higher in untreated ricottas, testifying greater deterioration's reactions. Since HPP ricottas at T3 respect all the limits of bacterial count permitted, it means that the treatment can ensure to this product a shelf life up to 30 days, more than the double guaranteed by untreated ones.

4. CONCLUSIONS

Food industry priorities in the Parmigiano Reggiano area are the creation of safe, nutritious and tasty dairy products with a long shelf life. For that reason, the High Pressure Processing (600 MPa, 5 min) was tested on ricottas, which were observed for a month in refrigerated storage at 4°C and compared with the same number of untreated ricottas. Observation and microbiological analyses show that HPP is able to slow down chemical reactions in treated ricottas, preserving their color and inhibiting the microbial growth up to 3 log CFU/g. In fact, modelling the total bacteria count and lactic acid bacteria growth in HPP ricottas with the Baranyi and Roberts primary model, it can be seen that the treatment prolongs the lag phase λ and reduce the maximum growth rate μ_{max} compared to untreated ricottas modelled with Baranyi and Roberts model without lag phase. Thanks to this, HPP ricottas have a shelf life of 30 days, while the untreated ones after 10 days of storage are already not suitable for consumers. However, it could be interesting to study the influence of pressure on microbial growth, analyzing different treatment parameters (as 400, 500 and 600 Mpa): of course, it will be the object of study in future developments of this work.

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APPENDIX

Figure 10: Lactic Acid bacteria in untreated ricottas modelled with Baranyi and Roberts equation (no lag)

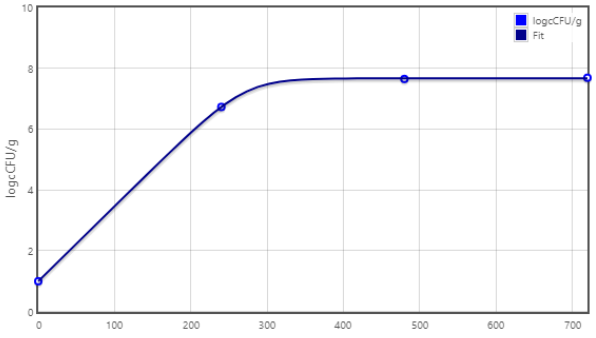


Figure 11: Nuclease negative cocci in untreated ricottas modelled with Baranyi and Roberts equation (no lag)

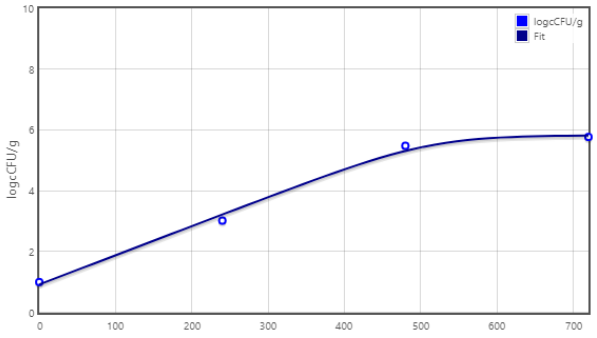
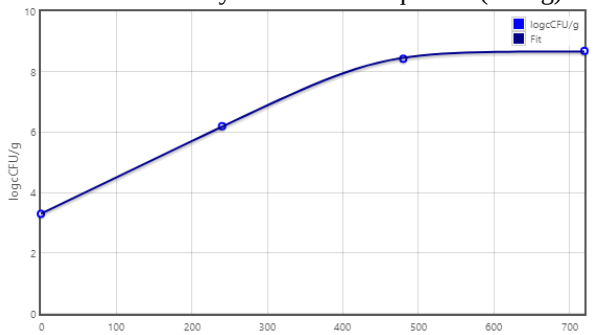


Figure 12: Total bacteria count in untreated ricottas modelled with Baranyi and Roberts equation (no lag)



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