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Quantitative Microbiological Analysis of Artisanal Stretched Cheese Manufacture

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Abstract: To evaluate the behavior of the relevant microbial populations during stretched cheese production, quantitative microbiological analysis was performed during the critical steps of the preparation. The obtained data distributions proved statistically significant increases in all indicators, on average by 4.55 ± 0.64 log CFU/g of presumptive lactococci counts, 4.06 ± 0.61 of lactobacilli, 1.53 ± 0.57 log CFU/g of coliforms, 2.42 ± 0.67 log CFU/g of *Escherichia coli*, 1.53 ± 0.75 log CFU/g of yeasts and molds, and 0.99 ± 0.27 log CFU/g of presumptive *Staphylococcus aureus*, from the early stage of milk coagulation until curd ripening (0–24 h). The following steaming/stretching process caused reductions in viable counts with the most significant inactivation effect on coliform bacteria, including *E. coli* (−4.0 ± 1.0 log CFU/g). Total viable counts and yeasts and molds showed 2 and almost 3 log reduction (−2.2 ± 1.1 log CFU/g and −2.6 ± 0.9 log CFU/g), respectively. The lowest decreases in presumptive *S. aureus* counts were estimated at the level of −1.50 ± 0.64 log CFU/g. The counts of yeasts and molds showed the best indicatory function during the entire storage period of vacuum-packaged cheeses at 6 °C.

Keywords: raw milk stretched cheeses; lactic acid bacteria; coliform bacteria; *Escherichia coli*; *Staphylococcus aureus*; yeasts and molds

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1. Introduction

The pasta filata cheeses share a unique processing step, when the curd is immersed in hot water or salt brine and mechanically stretched to a plastic consistency which can be formed or molded into a variety of shapes [1]. Some of this type of cheese are soft or semi-soft and usually consumed fresh or after a brief period of ageing (e.g., fresh Mozzarella, low-moisture Mozzarella, and Scamorza). Others, e.g., Caciocavallo, Kashkaval, Provolone, and Ragusano, are hard or semi-hard ripened cheeses with a considerable ageing period before consumption [2]. The history of some Slovakian artisanal types of steamed and stretched (pasta filata) cheeses can be observed in the Protections of Geographical Indications (PGI) of the following original agriculture products: Slovenský oštiepok, Slovenská parenica, Oravský korbáčik, Zázrivský korbáčik, and Zázrivké vojky. The records submitted to the PGI designations showed cooking, molding, or stretching processes as an integral part of traditional artisanal cheese manufacture have been used probably since the 17th to mid-18th centuries in the area of present Slovakia [3–7].

Industrial pasta filata cheese production uses acid-coagulated or fermented rennet curd from cow, sheep, goat, or buffalo pasteurized milk. The process technology includes two essential steps: cooking and stretching. Cooking is the phase in which the curd is transferred to the hot water section of the cooker/stretcher. Typical water temperatures vary between 60 °C and 75 °C, and cooked curd temperatures vary between 55 °C and 65 °C. Stretching describes the mechanical treatment of the curd following cooking [2].

On the other hand, traditional manufacture is based only on the artisanal cooking/stretching process of rennet curd made from raw milk [8]. Bacteria living in clusters

and incorporated in the casein network mostly concentrate in the curd during its formation, and the rest of the microbiota leaves this step with the whey [9]. Stretching of the raw milk cheese curd in hot water substantially effects the distribution and viability of the present microbiota; however, it does not destroy it completely [10,11]. Generally, the duration of the heat treatment is controlled empirically by cheese-makers based on the curd's melting and stretching properties [12]. In this context, many studies have pointed out the effect of the steaming and stretching phase on the microbial population of cheese curd, especially in relation to the presence of potentially pathogenic bacteria [13–16]. With current demands for milder food processing conditions, it is a challenge to apply a minimally required heat load while inactivating the target microorganisms sufficiently [17]. Thus, quantitative studies are needed to understand the behavior of the microbial population over the course of raw milk/cheese processing and to assess consumer exposure to undesirable microorganisms from this type of raw milk cheese.

That is why this study is focused on quantitative microbiological analysis in each processing step of raw milk steamed and stretched cheeses. The other aim was to provide a set of data for comparison with similar data from practice in order to help producers to improve the overall quality of these type artisan cheeses. In an effort to support the activity of native lactic acid bacteria (LAB), the culture Fresco DVS 1010 (Christian Hansen, Hørsholm, Denmark) consisting of Lactococcus lactis subsp. lactis, Lc. lactis subsp. cremoris and Streptococcus salivarius ssp. thermophilus was added prior to raw milk coagulation.

2. Materials and Methods

2.1. Cheese Preparation

Twenty steamed and stretched cheeses were prepared under laboratory conditions following the production steps described in the PGI documents [18,19]. Eighteen cheeses were produced using raw cow's milk (obtained from a vending machine in Bratislava, Slovak Republic) and two cheeses were produced using raw ewe's milk (provided by a local dairy farmer). The procedure of cheese production is schematically represented in Figure 1. Raw milk kept at 4–6 °C until the manufacture was heated to 30 ± 1 °C, inoculated with 1% (*v*/*v*) of overnight culture Fresco DVS 1010 (Christian Hansen, Hørsholm, Denmark), and coagulated using commercial rennet (FROMASE 220, DSM, Heerlen, Netherlands, strength 220 IMCU/mL) for 30–45 min. The small amount of the rennet (1.2 mL) enabled us to obtain the proper coagulate firmness to cut it and heat to 45 °C. The grains were collected with sterile cheese cloth and hung up to drip and ferment at ambient temperature (22–23 °C) until a pH of 5.1 (± 0.2) was reached. This step took 24 h, generally. Stretching of the curd was performed manually in hot water for 5 min with internal temperatures of 61–63 °C recorded using a data logger (Ebro EBI 100-T100, Ingolstadt, Germany). Temperature data has been processed with Winlog.pro 2.66 software (Xylem Analytics Germany Sales GmbH & Co., KG Ebro, Ingolstadt, Germany). For the storage tests, performed at 6 °C for five weeks, the final products were vacuum packed.

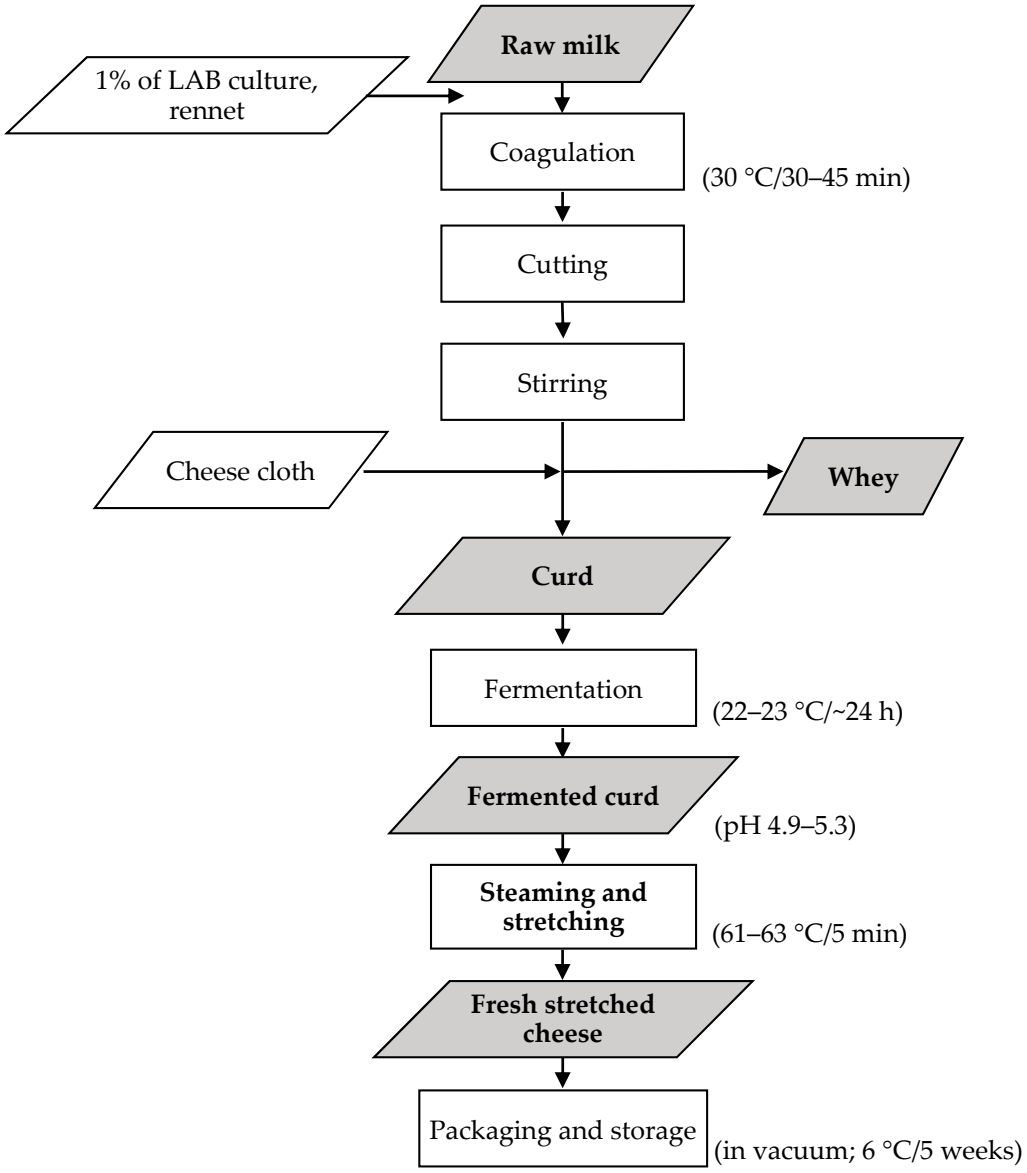


Figure 1. Flow diagram of the artisanal manufacture process applied in the preparation of steamed and stretched cheeses. The Fresco DVS 1010 (Christian Hansen, Hørsholm, Denmark) was used as LAB culture used in this work.

2.2. Microbiological Analysis

Samples of raw milk, curd after coagulation, curd before and after the stretching process, and cheese at different storage times (1, 2, 3, 4, and 5 weeks) were taken, and 10-fold dilutions according to ISO 6887-5:2010 [20] were prepared. The following microbial groups were determined: the presumptive counts of lactococci (pLC) were determined on M17 agar (Biolife, Milan, Italy) according to EN ISO 15214 [21], with aerobic incubation at 30 ± 0.5 °C for 48 h; lactobacilli (LBC) were determined according to [21] on MRS agar (Biokar Diagnostics, Beauvais, France) with anaerobic (5% CO_2) incubation at 37 ± 0.5 °C for 72 h; coliform bacteria (CFM) and *Escherichia coli* (EC) on Chromogenic Chromocult coliform agar (Merck KGaA, Darmstadt, Germany) incubated aerobically at 37 ± 1 °C for 24 h [22]; presumptive counts of *Staphylococcus aureus* (pSTA) on selective Baird-Parker medium (Merck KGaA, Darmstadt, Germany) incubated aerobically at 37 ± 1 °C for 48 h [23]; total viable count (TVC) was determined on Plate Count Agar (Biokar Diagnostics, Beauvais, France) incubated aerobically at 30 ± 1 °C for 72 h [24]; and yeasts and moulds

(Y&M) on Yeast Glucose Chloramphenicol agar (Biokar Diagnostics, Beauvais, France) incubated aerobically at 25 ± 1 °C for 72–120 h [25].

2.3. Additional Analysis

During cheese-making trials, the pH and titratable acidity were determined. The pH value of curd before the stretching process was measured using a WTW 720 pH-meter (Inolab, Weilheim, Germany) with a Sen Tix 81 glass electrode (WTW GmbH, Weilheim, Germany). The titratable acidity (°SH) was manually determined by visual titration with 0.25 M NaOH according to Calamari et al., 2016 [26].

2.4. Statistical Analysis

Statistical analysis of the results was performed using the statistical analysis package Analyse-it for Microsoft Excel, Method Validation Edition version 5.66 (Analyse-it Software, Ltd., Leeds, UK). The statistical significance of differences in the means of microbial counts was evaluated by using an ANOVA model. When differences were significant ($p < 0.05$), the means were compared using Tukey's post-hoc test. Fitting of the data distribution was performed using the Risk Analysis Add-in for Microsoft Excel @RISK version 7.5.1 (Palisade Corp., Ithaca, NY, USA).

3. Results

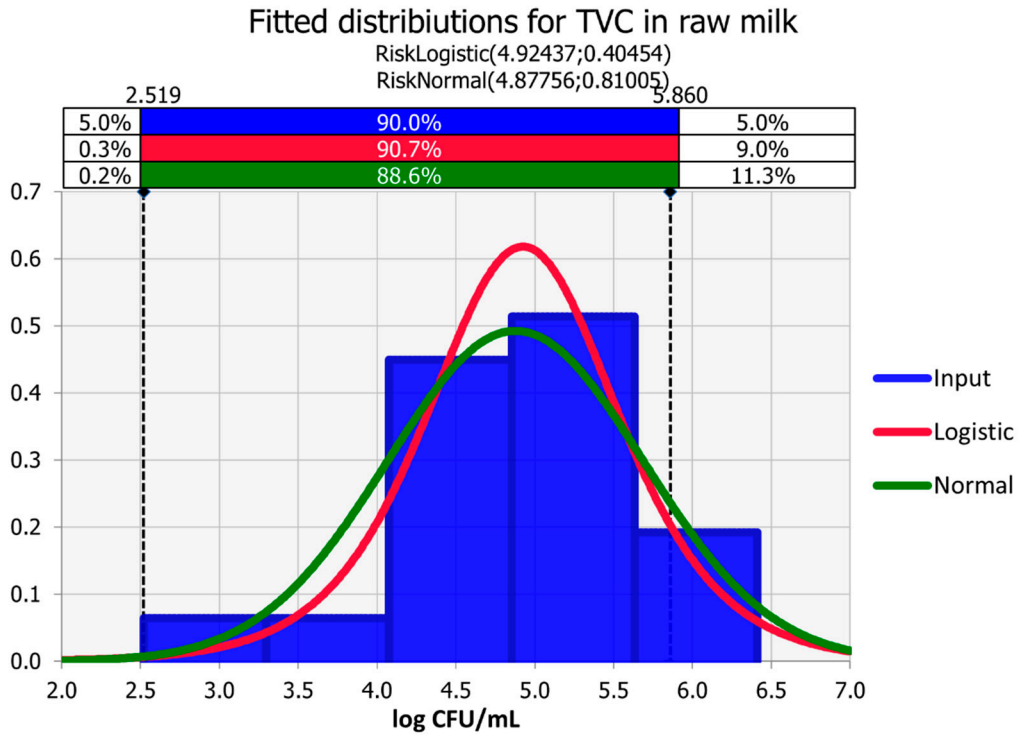
The traditional steamed and stretched cheeses Slovenský oštiepok, Slovenská parenica, Oravský korbáčik, Zázrivský korbáčik, and Zázrivské vojky are favourite artisanal products of Slovakia that have obtained legal EU PGI recognition [3–7]. In their original versions, they are currently produced only by small producers and are distributed only locally or regionally.

This work provides the data distributions and variabilities of microbiological indicators of 20 raw milk curds steamed and stretched according to an artisanal procedure during laboratory preparation. They include the counts of lactococci (pLC) and lactobacilli (LBC), total viable count (TVC), coliforms (CFM), *E. coli* (EC), presumptive *S. aureus* (pSTA), and yeasts and molds (Y&M) at each relevant manufacturing step: in raw milk, fresh cheese curd after coagulation, whey, cheese curd after 24 h of fermentation, before the steaming and stretching, and in fresh stretched cheese. Similar microbial indicator datasets (n = 13) resulted from a five-week storage period of cheeses.

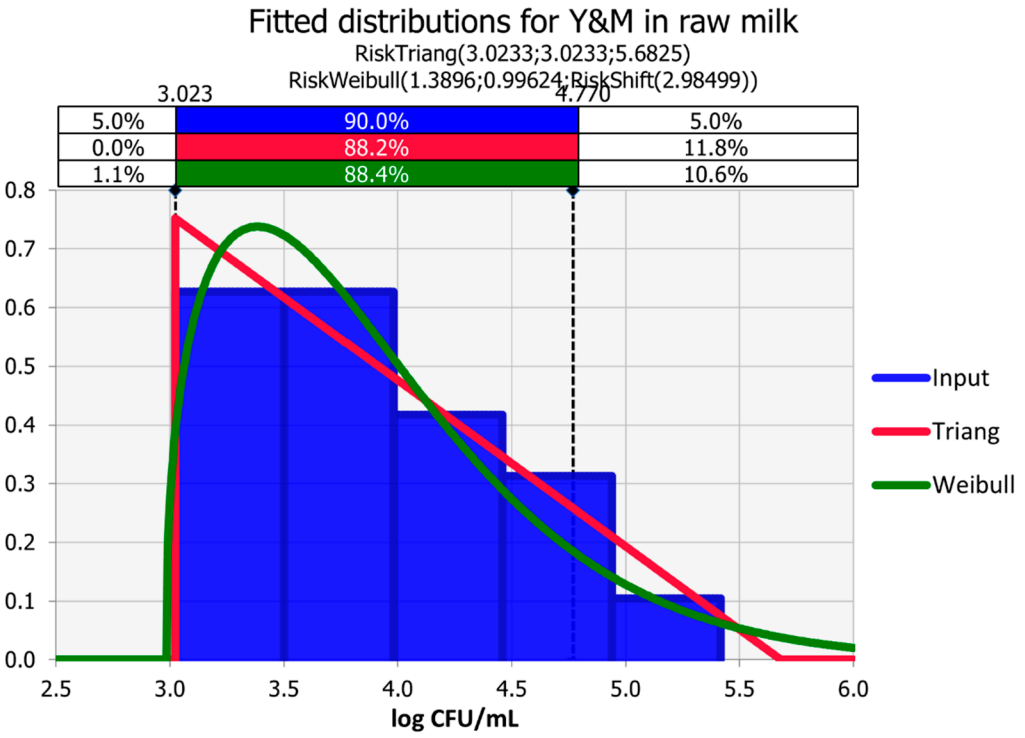
The outputs of this work not only provide specific data for a small part of artisanal cheese practice producing cheeses with PGI but can also be helpful for common pasta filata cheese practice. On the other hand, the statistically evaluated data can be used for validating models of *E. coli* and *S. aureus* behavior, as well as assessing exposure from consumption of this type of cheese. Moreover, applying the concept of a Food Safety Objective [27], the steps associated with the increase (I_G) or reduction (R) of microbial counts are identified in order to provide performance criteria for the artisanal production steps, including the initial microbial load in raw material (H_0).

3.1. Raw Milk

The microbiological quality of the raw milk used in our work was characterized by the following average values and standard deviations: 4.6 ± 0.3 log CFU/mL for lactococci, 4.1 ± 0.8 log CFU/mL for lactobacilli 4.9 ± 0.5 log CFU/mL for TVC, 2.6 ± 0.2 log CFU/mL for coliforms, and 3.9 ± 0.6 log CFU/mL for Y&M (Table 1). The pSTA counts of 2.97 ± 0.55 log CFU/mL were higher than those of *E. coli* that were determined over limit of the detection in 18 samples, averaging 1.5 ± 0.7 log CFU/mL. Taking the Akaike Information Criterion (AIC), the best statistical indices provided fitting with logistic distribution in the case of TVC, coliforms, pSTA and triangle distributions for Y&M and EC (Figure 2). Since the pLC and LBC have been determined only in three cases of preparation the data distribution could not be provided.

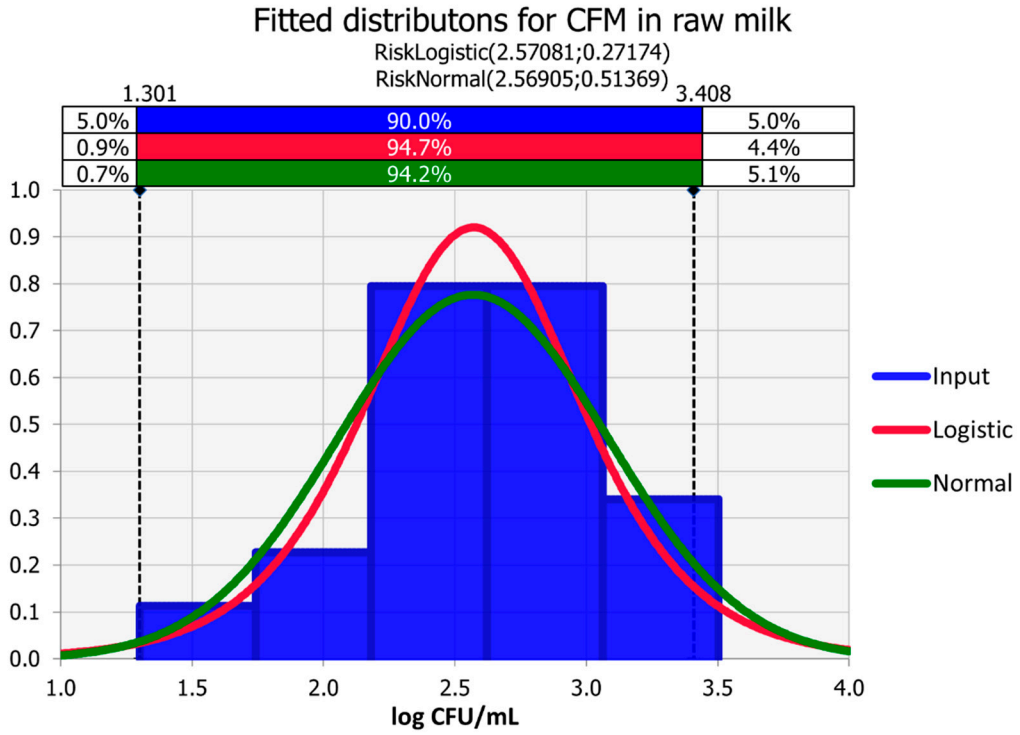


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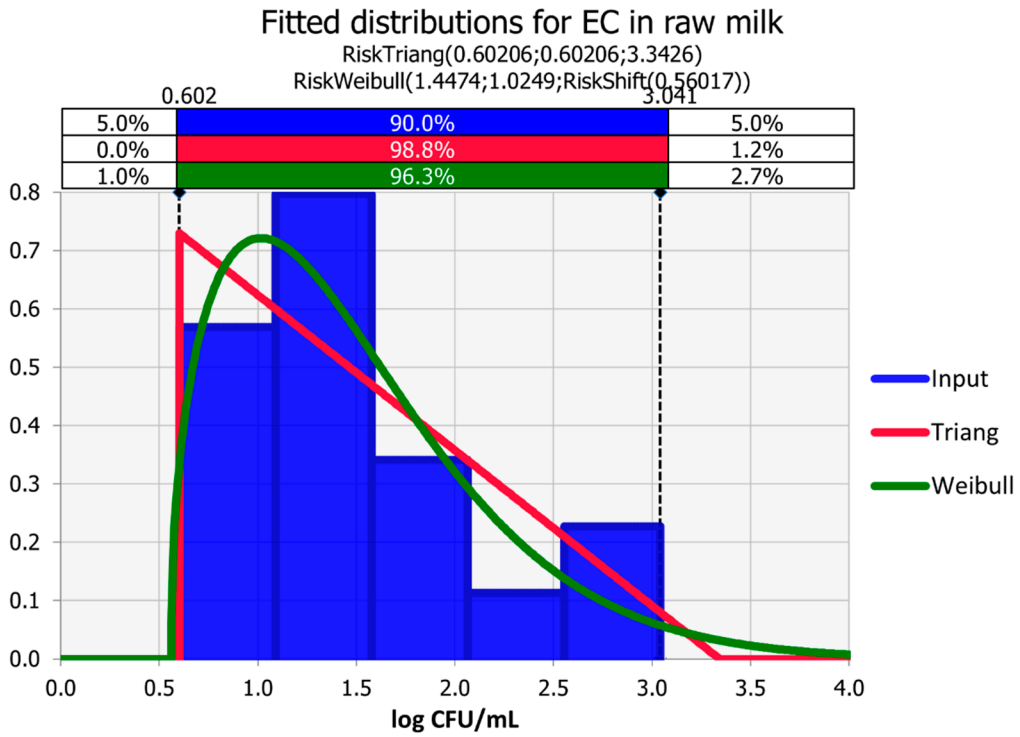


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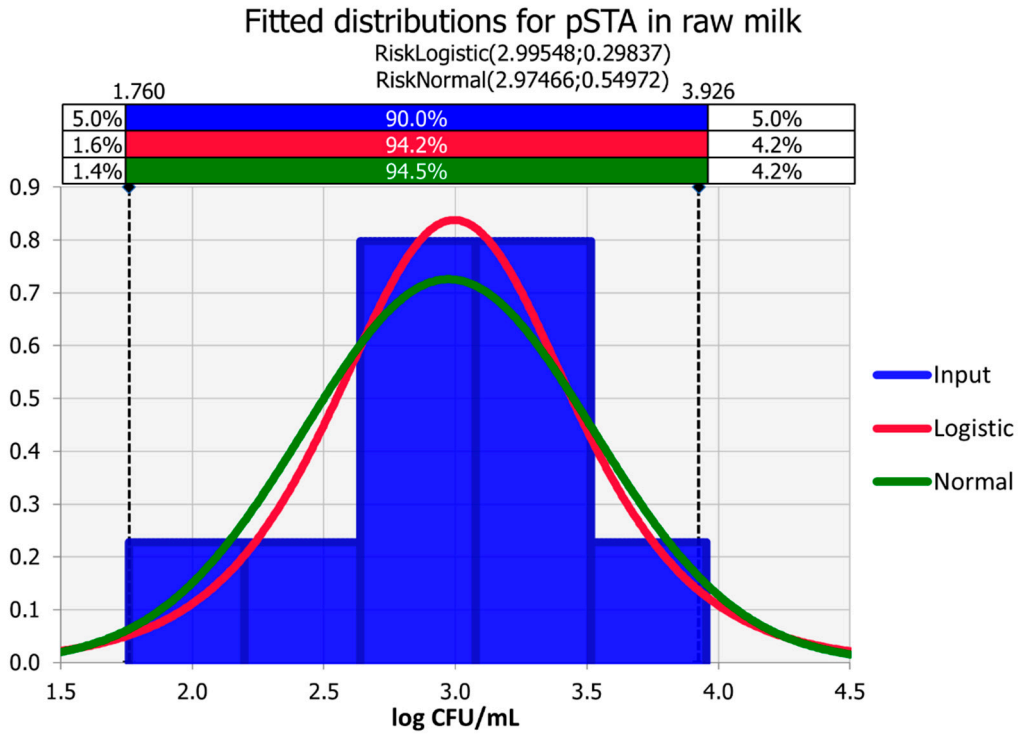


(**c**)



(**d**)

Figure 2. *Cont.*



(**e**)

Figure 2. Fitted distributions for microbiological indicators in raw milk: (**a**) total viable count (TVC); (**b**) yeasts and molds (Y&M); (**c**) coliforms (CFM); (**d**) *Escherichia coli* (EC); (**e**) presumptive *Staphylococcus aureus* (pSTA).

Table 1. The average counts of microorganisms determined in the cheeses produced from raw milk at different processing steps [1].

Microbial Group	Raw Milk	Fresh Curd	Whey	Fermented Cheese Curd (Before Stretching)	Stretched Cheese
	[log CFU/mL]	[log CFU/g]	[log CFU/mL]	[log CFU/g]	
Total viable count	4.88 (±0.50)	7.18 (±0.69)	6.58 (±0.38)	9.36 (±0.64)	7.44 (±0.98)
Presumptive lactococci [2]	4.55 (±0.25)	6.89 (±0.66)	6.29 (±0.92)	8.74 (±0.44)	5.50 (±0.56)
Lactobacillus sp. [2]	4.06 (±0.75)	6.68 (±0.41)	6.16 (±0.38)	8.81 (±0.24)	4.34 (±0.41)
Coliforms	2.57 (±0.20)	2.91 (±0.45)	2.27 (±0.37)	4.99 (±0.24)	1.02 (±0.50)
E. coli	1.49 (±0.54)	1.82 (±0.50)	0.73 (±0.49)	3.01 (±0.53)	0.64 (±0.81)
Presumptive *S. aureus*	2.97 (±0.79)	3.19 (±0.39)	2.45 (±0.37)	3.97 (±0.14)	2.46 (±1.27)
Yeasts and molds	3.90 (±0.64)	4.43 (±0.69)	3.70 (±0.69)	5.43 (±0.71)	2.81 (±0.64)

[1] These results of 20 replicate cheese-making trials (n = 20) are expressed as the average value ± standard deviation. [2] Average of three replicates.

3.2. Coagulation

In the coagulation step, the microbial population partitioning between the curd and whey was examined. The results are presented in Figure 3 as the whey/curd index (W/C in %), which represents the average percentage value of individual viable counts determined in whey (in CFU/mL) from the counts in curd (in CFU/g). Generally, it seems that approximately less than half of the population was removed from curd to whey within the syneresis. In detail, for example, 70 ± 16% of the TVC population remained in the curd. Expressed as log values, this means that on average, the counts determined in whey were lower by 0.6 (TVC) to 1.0 (EC) log CFU/mL as compared to the log counts in curd. Testing paired data of indicators determined in whey and curd via ANOVA showed statistically significant differences between the datasets in all cases of microbial indicators at the level of p = 0.05.

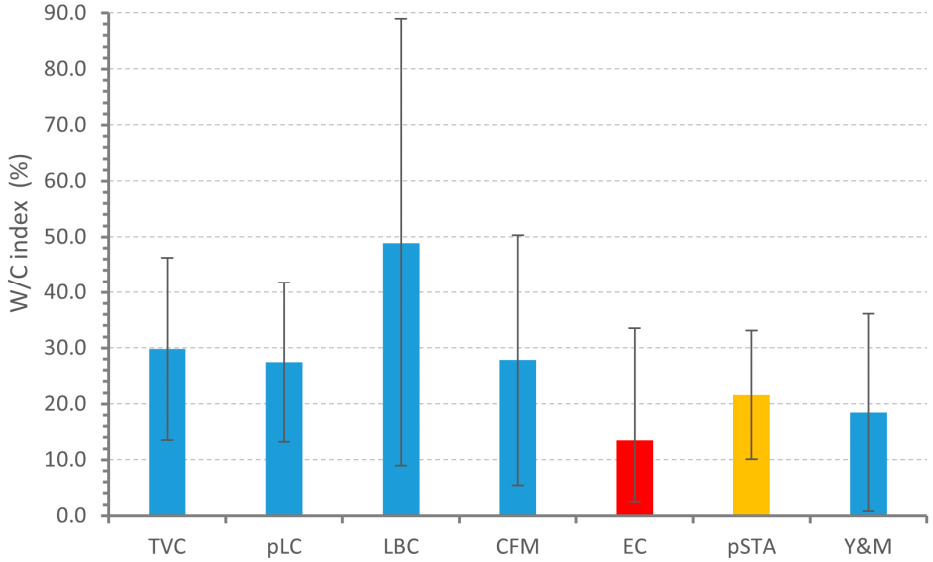


Figure 3. The microbial population partitioning between whey (W) and curd (C). The values of pLC and LBC represent only 3 replications.

3.3. Curd Fermentation

From the early stage of milk coagulation until curd ripening (0–24 h), the increases in microbial numbers due to growth (I_G) are shown in Figure 4. Testing paired data via ANOVA confirmed statistically significant I_G values for all microbiological indicators during the 24 h of curd fermentation. Thus, the lactococci and lactobacilli increased on average to almost 9 logs CFU/g, while Y&M, CFM, EC, and pSTA increased to 5.4 ± 0.7 log CFU/g, 5.0 ± 0.2 log CFU/g, 3.0 ± 0.5 log CFU/g, and 4.0 ± 0.1 log CFU/g, respectively. The fermentation of cheese curds at 21 ± 0.5 °C for 24 h resulted in a final pH of 5.1 and titratable acidity of 81 °SH.

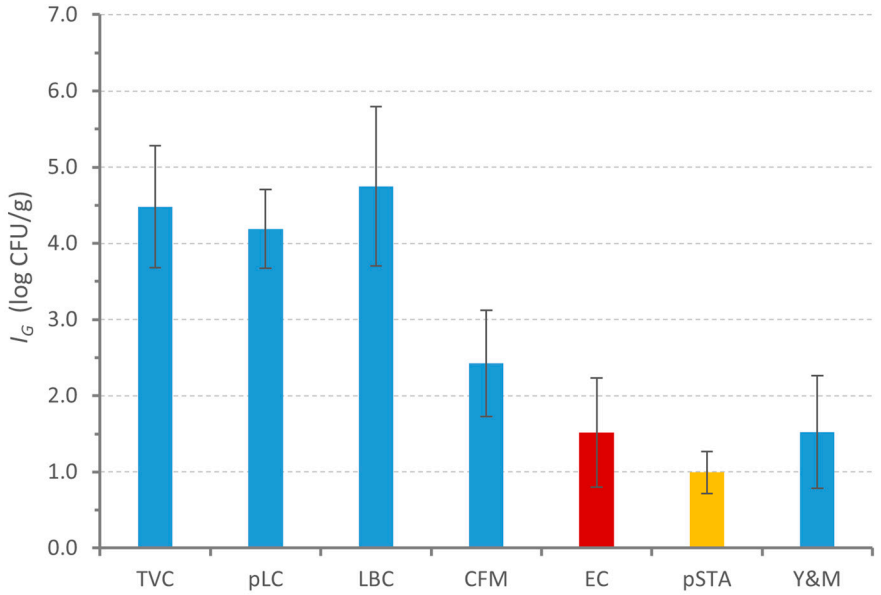
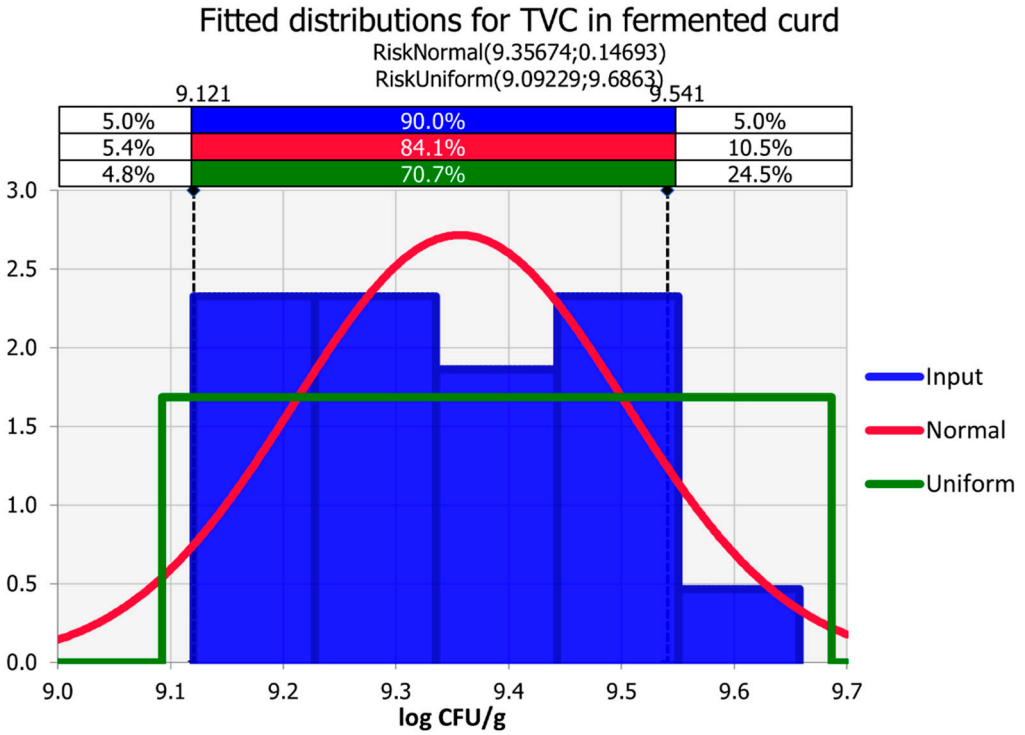


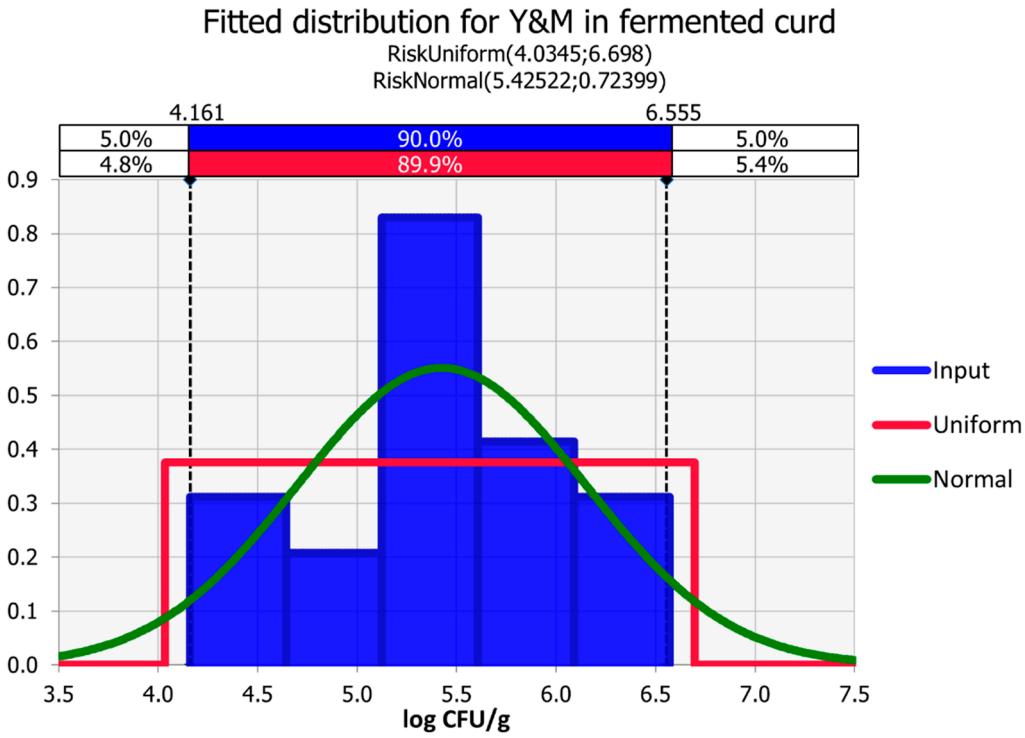
Figure 4. Increase in the counts of microbial indicators (IG) as a result of their growth during 24 h of curd fermentation at 22–23 °C.

Data representing the microbiological quality of the cheese curds prior to steaming and stretching were quantitatively described with the uniform and normal distributions (Figure 5). Except for the number of data points at each indicator (n = 20), it seems that these

distributions reflected the stationary phase, in which the microbial populations appeared most likely in this step.

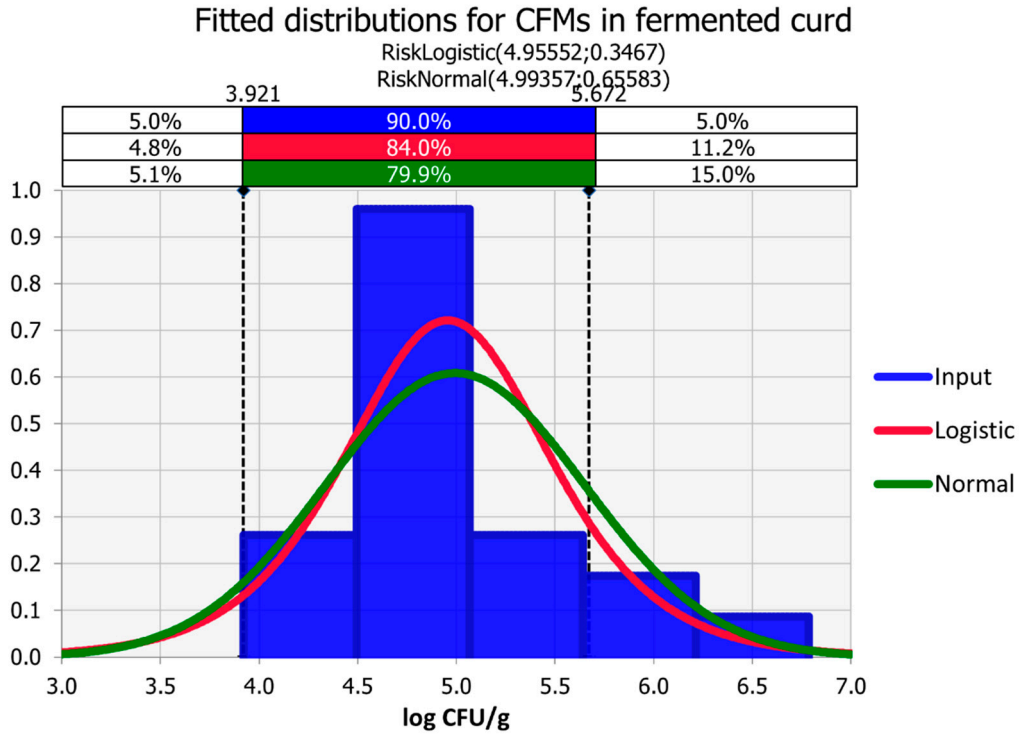


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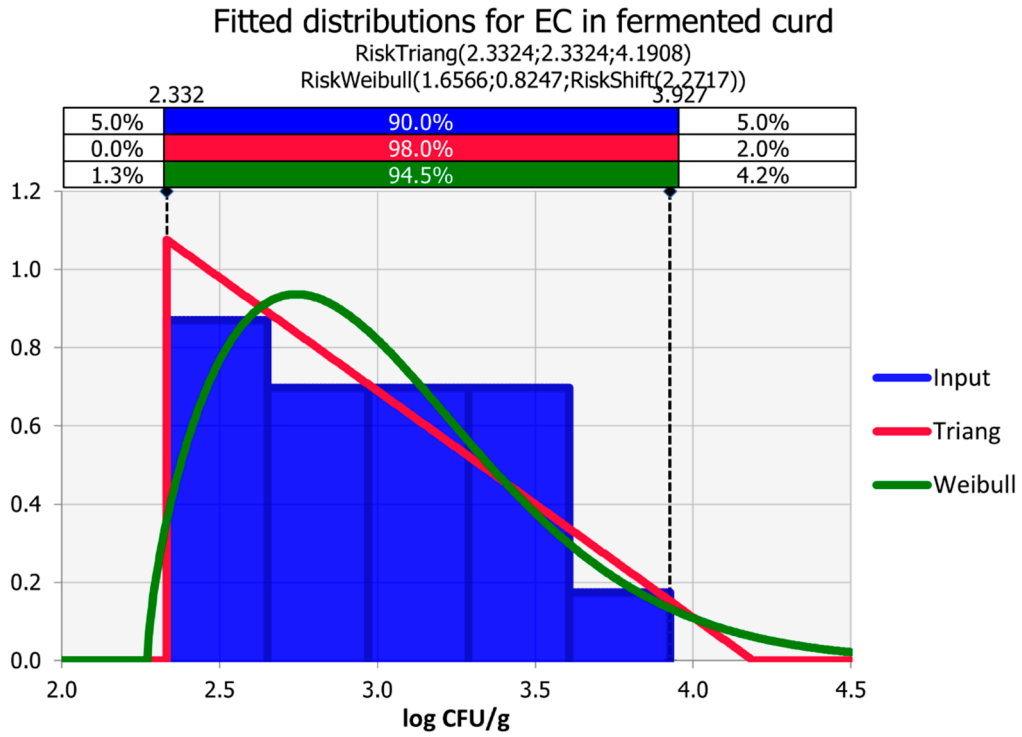


(**b**)

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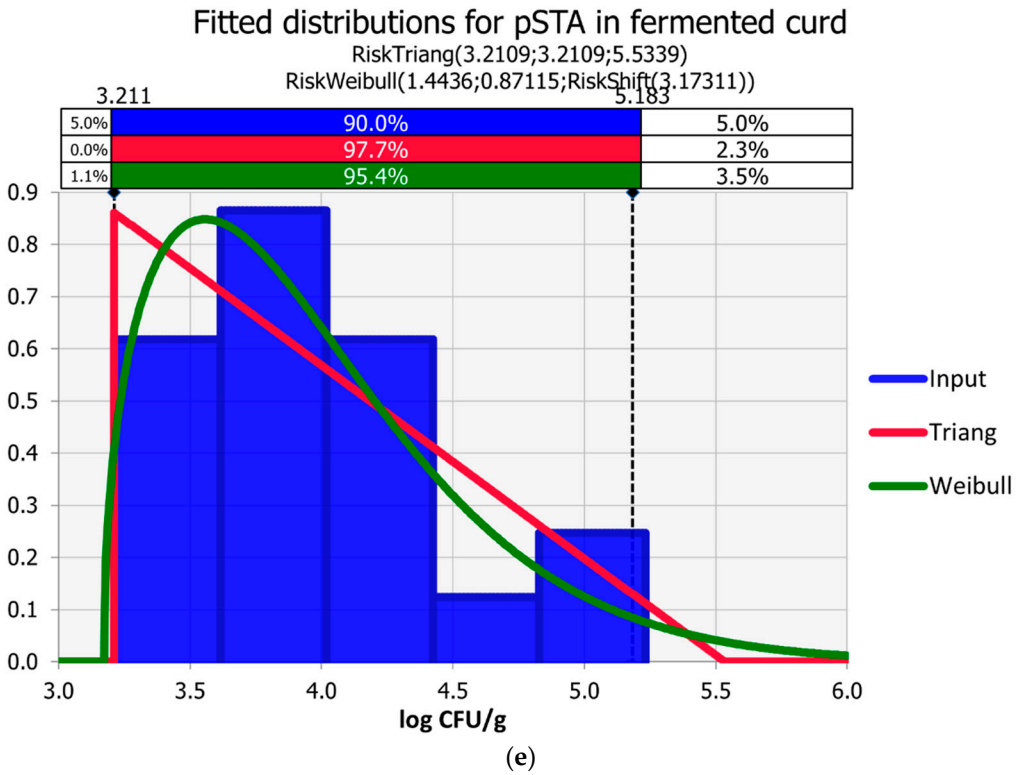


Figure 5. Fitted distributions for microbiological indicators in fermented curd: (**a**) TVC; (**b**) Y&M; (**c**) CFM; (**d**) EC; (**e**) pSTA.

3.4. Steaming and Stretching Process

Steaming and stretching of the curds were performed in hot water at an average internal curd temperature of 62.2 ± 1.4 °C (n = 481; the data were measured and recorded by a data logger in 15 s intervals) for 4.5 min ± 0.9 min (n = 20). This is the only process in the cheese manufacture aimed at the inactivation of undesirable microorganisms. Significant reductions (R) in viable microbial counts were observed in steamed curd (Figure 6) with the highest average LBC and CFM counts 4.5 ± 0.6 and 3.8 ± 1.0 log CFU/g, respectively, followed with lactococci and Y&M showing the level of 3 log reductions (3.1 ± 0.7 log CFU/mL and 2.6 ± 0.9 log CFU/mL, respectively). In some cases, the counts of EC could not be determined by cultivation methods, however, their reductions were estimated at a level of approximately 2.3 ± 0.8 log CFU/g. The lowest inactivation rate was found in pSTA counts and ranged from 0.53 to 2.87 log CFU/g with average value 1.5 ± 0.6 log CFU/g. Importantly, CFM counts over the detection level of 150 CFU/mL were determined only in 5 samples of the 20 steamed and stretched cheeses produced in the laboratory, thus CFM and EC were present in very low concentrations of 1.0 and 0.6 on average, respectively.

3.5. Storage Test

The temporal evolution of the microbiological quality indicators of the final cheeses during five weeks of storage is reported in Figure 7. First, statistically significant increases were confirmed between the counts in Week 0 and Week 5 for TVC, Y&M, and pSTA during storage tests of vacuum-packaged cheese samples at 6 °C (n = 13). In the case of lactobacilli, their average counts calculated only from 3 replicates showed negligible increase in 0.3 log CFU/g during 5 weeks storage, while the counts of lactococci decreased by 0.5 log CFU/g. The other average values of indicators increased in 0.9 ± 1.0 log CFU/g, 0.9 ± 0.5 log CFU/g, and 3.1 ± 1.3 log CFU/g for TVC, Y&M, and pSTA, respectively. However, this was not the case for CFM and EC, as they were estimated to be mostly under the detection level (less than 150 CFU/g); moreover, countable EC colonies were found in 4% samples. The coliforms (with a maximal average of 1.5 ± 0.7 log CFU/g in the second

week), including EC, did not reach 2 log, and pSTA did not exceeded 3 log during the entire period of storage. On the other hand, the counts of pSTA were determined in 90% of the cheese samples during the storage period. Despite vacuum packaging, Y&M were identified as the most relevant microbiological quality indicator with I_G > 3 log on average between Week 0 and Week 5.

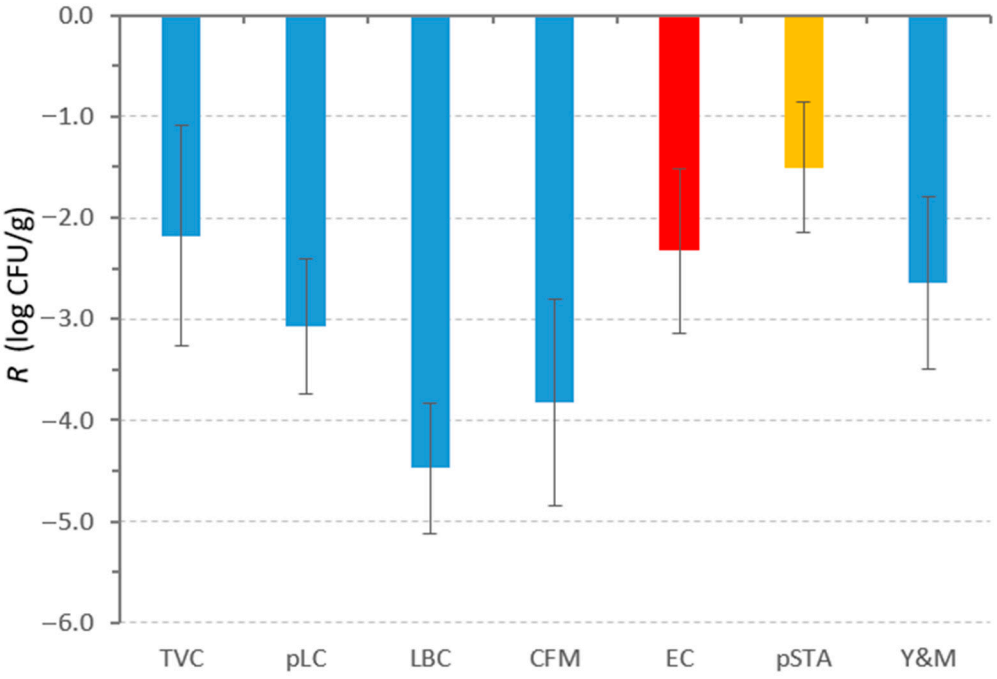


Figure 6. Reductions in the microbial indicators during the steaming and stretching process. The values of pLC and LBC represent only 3 replications.

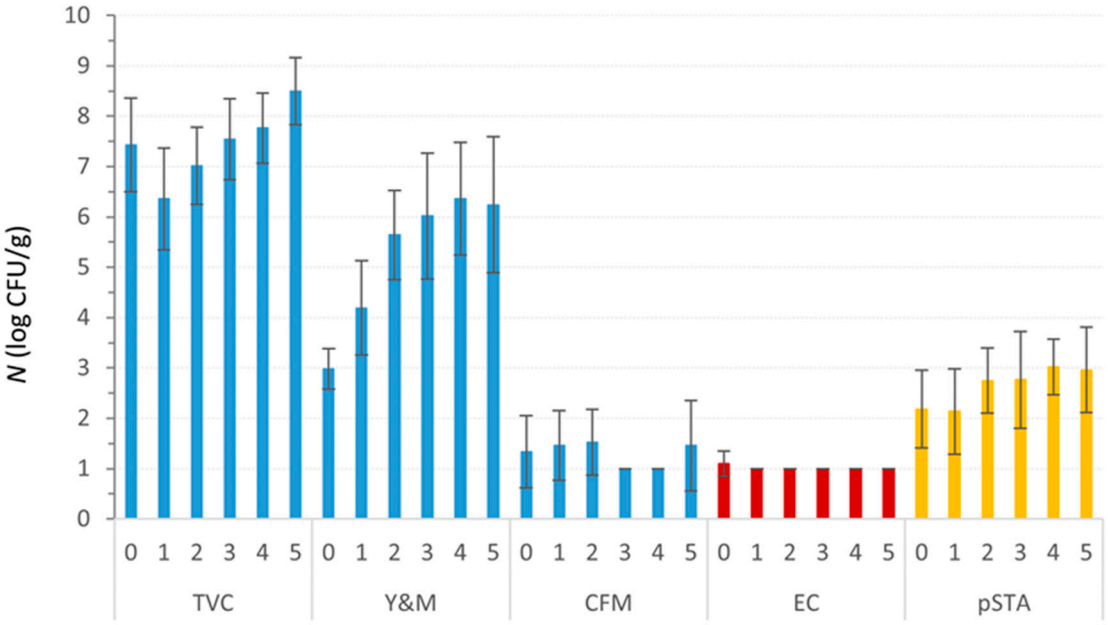


Figure 7. The microbial indicator counts in vacuum-packaged steamed and stretched cheese (n = 13) stored at 6 °C for five weeks.

4. Discussion

Cheese production belongs to the largest sectors of the dairy and food industries, and much of it is still realized in an artisanal environment using raw milk [28]. While long-ripened cheeses produced under hygienic conditions are not the frequent subject of microbiological quality or safety concerns, the fresh, soft, or short-ripened raw milk cheeses are the subjects of potential health risk discussions. Despite the history and considerable expansion of the artisanal production of steamed and stretched cheeses in Slovakia, there is a lack of data not only on the volumes produced but also on the consumption of this kind of raw milk cheeses, as the relevant consumption is hidden within the group of soft cheeses at approximately 2 kg/person/year [29]. Towards preliminary exposure assessment, microbiological data during their production and storage are needed. That is why this study deals with the quantitative changes of microbial indicators in the relevant steps of the manual production of raw milk steamed and stretched cheese, thus contributing to discussions about the quality and safety of traditional Slovakian cheeses.

It is well known that the first production steps, including curd fermentation, are the most critical for potential microbial growth of undesirable microorganisms, here represented via indicators as well as the counts of *E. coli* and *S. aureus* [9,13,30,31]. Importantly, the proliferation of spoilage or pathogenic microorganisms at these early steps should be effectively limited by a starter culture of lactic acid bacteria (SLAB). Their inhibitory effects are linked to the rate of lactose fermentation or lactic acid production, the associated decrease in pH, and finally their growth competitive abilities [32,33]. Additionally, SLAB can produce other antimicrobial metabolites, such as bacteriocins and non-protein organic compounds [34,35]. To ensure consistency of the fermentation process, the SLAB need to be active and dominant in the curd. That is why the mesophilic culture Fresco DVS 1010 was used in this work. In this way, LAB counts close to 9 log CFU/g were reached within the first 24 h. Based on data from competitive growth modelling among three populations (LAB, *S. aureus*, and *E. coli*), an initial number of LAB at 5 log CFU/mL was able to bring the populations of *S. aureus* and *E. coli* under control, reaching numbers higher than 8 log CFU/g at 30 °C after 6 h, while at initial 6 log CFU/mL of LAB, 8 log CFU/g was reached after 4 h [36]. Charlier et al. [34] pointed also to the ratio between *S. aureus* and SLAB as a determining parameter for the efficacy of *S. aureus* inhibition in the cheese matrix [33]. Thus, prior to steaming and stretching, the counts of *E. coli* and *S. aureus* in 24 h curd remain between 10^3 and 10^4 CFU/g.

However, the question remains in this study of the extent to which the manually performed steaming and stretching process inactivates the populations in the curd. It seems that the results in shown in Figure 6 and the subsequent microbiological storage data at 6 °C (Figure 7) confirm that the inactivation step sufficiently reduces the microbial population and keeps CFM, *E. coli*, and *S. aureus* under control. On the other hand, according to Onipchenko et al. [15] and Samelis et al. [16], the coliforms can be reduced by up to 4 log CFU/g in heat-treated curd. On the contrary, but in accordance with Mangia et al. [37], an *S. aureus* population was present in all prepared fresh pasta filata cheeses, probably due to their tolerance to mild heat treatment [36] or to secondary contamination, as this is not possible to exclude during manual preparation. For example, Samelis et al. [16] reported 99.7% mean inactivation of total staphylococci after stretching the curd at ~80 °C during Kashkaval "pasta filata" cheese production [16]. Fuentes et al. [38] showed a significant decrease ($p < 0.05$) in coliform bacteria, including *E. coli*, in Oaxaca "Mexican pasta filata" cheese kept under vacuum conditions at 8 °C for 24 days [38], and consistent with our results, Todaro et al. [39] did not observe any increase in coliform and *E. coli* counts during the entire storage period (180 days). In addition, the coagulase-positive staphylococci were kept below the detection limit.

As total viable counts at the level of 7 log CFU/g remained in cheeses after steaming, stretching, and cooling, they also contributed to the control of other populations, except Y&M. Thus, only this eukaryotic population was able to increase its counts above 3 log CFU/g even at vacuum conditions during the five-week storage period at 6 °C. However,

they were kept under 10^6 CFU/g in the cheeses for two weeks. Based on this knowledge and taking the inhibitory potential of LAB and other competitive microbiota into account, the shelf-life of artisanal raw milk stretched cheeses should be defined as less than 14 days. Modification of temperature and time combination of the steaming and stretching process during artisanal methods of manufacture is extremely limited. With respect to D_{60}-values of 5–6 min and z-values of about 8 °C for *S. aureus* and *E. coli*, determined at 60 °C by Kennedy et al. [40] and Li and Gänzle [41], the time and temperature of the process should be prolonged to more than 5 min or the temperature kept higher than 78 °C to decrease the numbers of these bacteria by another 1 log CFU/g. Applying such conditions is physiologically impossible in manual production. However, these options can be realized in industrial pasta filata cheese production. For this reason, future work will be focused on quantitative modelling and simulation of bacterial behavior in artisanal steamed and stretched cheeses and assessing the exposure of consumers to *E. coli* and *S. aureus* from their consumption.

5. Conclusions

This study provides a coherent view on the behavior of microorganisms during the artisanal production of Slovakian fermented, steamed and stretched cheeses. The obtained data point to a potential hazard of microbial growth in early stage of milk and curd fermentation, despite the mild heat treatment of the curd is incorporated into this manufacturing method. Therefore, it is necessary to know the relations between microbiological quality and safety data and artisanal manufacturing conditions, including the efficacy of critical process steps. Relevant data from this work can then be used to assess or validate the potential exposure to *E. coli* or *S. aureus* from the consumption of artisanal stretched cheese.

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