

## MICROBIOLOGICAL QUALITY AND REGULATORY SAFETY ASSESSMENT OF FRESH CHEESE ENRICHED WITH MINT AND THYME

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**Abstract:** This study evaluated the microbiological quality and regulatory safety of fresh cow's-milk cheese produced without herbs (control) and with mint (*Mentha piperita* L.) or thyme (*Thymus vulgaris* L.) during 20 days of storage. Lactic acid bacteria (LAB), *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, yeasts and moulds, anaerobic bacteria and Enterobacteriaceae were enumerated, while *Salmonella* spp. was assessed qualitatively. Analyses were performed on days 1, 7, 12 and 20, generally in duplicate. Initial LAB counts were higher in the mint and thyme cheeses (5.26 and 4.99 log<sub>10</sub> CFU/g, respectively) than in the control (3.74 log<sub>10</sub> CFU/g). LAB decreased to day 12 in both herb-enriched cheeses and partially recovered by day 20, whereas the control showed a transient increase on day 12. Yeasts and moulds increased markedly on day 7 in all variants, reaching 4.33, 4.48 and 5.08 log<sub>10</sub> CFU/g in control, mint and thyme cheese, respectively, followed by lower counts on days 12 and 20. *E. coli*, anaerobes and *Salmonella* spp. were not detected throughout storage. Low, sporadic counts of *S. aureus* and *B. cereus* were observed, and Enterobacteriaceae were restricted to early storage. The results were below the relevant numerical limits of the national microbiological criteria for *Salmonella* and coagulase-positive staphylococci. However, because analytical duplicates from one production batch do not constitute the statutory sampling plan, the findings support an indicative safety assessment rather than a formal declaration of legal conformity. Herb addition modified microbial succession but did not uniformly suppress LAB or yeasts and moulds.

**Keywords:** fresh cheese, *Mentha piperita*, *Thymus vulgaris*, lactic acid bacteria, yeasts and moulds, food safety

### 1. INTRODUCTION

Fresh cheese is a high-moisture dairy product with a short shelf life and a microbiota that can change rapidly during storage. Its microbial ecology reflects the initial quality of milk, processing hygiene, acid development, salt concentration, temperature and post-production handling. Lactic acid bacteria are technologically desirable because they contribute to acidification and may inhibit undesirable microorganisms, while yeasts and moulds can become important spoilage agents when oxygen, moisture and suitable substrates are available (Fox et al., 2017; D'Amico, 2014).

Aromatic herbs are increasingly used to diversify traditional dairy products and to provide naturally derived bioactive compounds. Mint and thyme contain phenolic constituents and volatile compounds with documented antimicrobial potential, although their effect in a complex food matrix depends on concentration, microbial species, fat and protein interactions and storage conditions (Burt, 2004; Gyawali and Ibrahim, 2014). Consequently, inhibition observed in vitro cannot automatically be assumed to occur in cheese.

A recent refrigerated fresh-cheese study likewise demonstrated that dried mint and thyme and their essential oils can alter LAB, coliform and fungal populations, while the magnitude of inhibition depends on the form of the plant material and the storage system (Kačaniová et al., 2024).

The objective of this study was to compare the microbiological dynamics of control fresh cheese and cheeses enriched with mint or thyme over 20 days and to assess the observed values against the microbiological criteria applicable in the Republic of North Macedonia, with particular emphasis on food-safety criteria, process-hygiene indicators and the limits of regulatory interpretation.

### 2. MATERIALS AND METHODS

Three variants of fresh cow's-milk cheese were examined: cheese without herbs (control), cheese with mint (*M. piperita* L.) and cheese with thyme (*T. vulgaris* L.). The products were prepared within the same experimental production cycle. The cheese was manufactured by acid coagulation of cow's milk; 40 mL of 9% acetic acid per

litre of milk was used as the coagulant, (Dimitrovska and Makarijoski, 2021) The herb variants were obtained by incorporating mint or thyme into the curd, while the control was produced without herbs. Samples were monitored during a 20-day storage period.

Microbiological analyses were performed on days 1, 7, 12 and 20. The investigated parameters were LAB, *E. coli*, *S. aureus*, *B. cereus*, yeasts and moulds, anaerobic bacteria and Enterobacteriaceae, expressed as colony-forming units per gram (CFU/g). *Salmonella* spp. was reported as detected/not detected. Culture-based enumeration and qualitative detection procedures were used. Most determinations were conducted in duplicate; results of zero CFU/g were recorded as zero for both replicates. For thyme-cheese LAB on day 20, three reported determinations were available.

Regulatory interpretation was based on the Rulebook on special requirements relating to microbiological criteria for food (Official Gazette of the Republic of Macedonia No. 100/2013), including the amendments published in Nos. 145/2014, 37/2017, 173/2018, 229/2020 and 287/2022. The framework is aligned with the risk-based structure of Commission Regulation (EC) No 2073/2005. Results were compared with the criteria relevant to cheese made from raw milk: absence of *Salmonella* in 25 g and the process-hygiene criterion for coagulase-positive staphylococci ( $n = 5$ ,  $c = 2$ ,  $m = 10,000$  and  $M = 100,000$  CFU/g). Other enumerated groups were interpreted as technological, spoilage or supplementary hygiene indicators where no product-specific statutory limit applied.

Duplicate plate counts were summarized as arithmetic mean  $\pm$  standard deviation. To facilitate comparison across several orders of magnitude, mean counts were also transformed to log<sub>10</sub> CFU/g. Zero counts were not logarithmically transformed and are presented as not detected. Because the dataset represents one production trial with analytical replicates rather than independent production batches, inferential significance testing was not applied.

### 3. RESULTS AND DISCUSSION

The evolution of LAB and yeasts and moulds is summarized in Table 1 and Figure 1. On day 1, LAB were considerably more abundant in the herb-enriched cheeses than in the control. Mint cheese showed the highest initial mean ( $180,000 \pm 28,284$  CFU/g;  $5.26 \log_{10}$  CFU/g), followed by thyme cheese ( $98,500 \pm 2,121$  CFU/g;  $4.99 \log_{10}$  CFU/g) and the control ( $5,500 \pm 424$  CFU/g;  $3.74 \log_{10}$  CFU/g). Raw-milk cheeses commonly harbour heterogeneous microbial communities derived from milk, the processing environment and added plant materials (Quigley et al., 2013; Montel et al., 2014). Accordingly, the higher initial LAB counts in the herb variants may reflect herb-associated microbiota, differences in early acidification or matrix interactions. They should not be

interpreted as proof of a stimulatory herb effect because independent production replicates were not available.

**Table 1. Mean microbial counts during storage.**

| Period  | LAB(log 10 CFU/g) |       |        |        | Yeast and molds (log 10 CFU/g) |       |        |        |
|---------|-------------------|-------|--------|--------|--------------------------------|-------|--------|--------|
|         | Day 1             | Day 7 | Day 12 | Day 20 | Day 1                          | Day 7 | Day 12 | Day 20 |
| Variant |                   |       |        |        |                                |       |        |        |
| Control | 3.74              | 3.46  | 4.14   | 3.49   | 2.08                           | 4.33  | 4.36   | 3.51   |
| Mint    | 5.26              | 4.37  | 3.48   | 4.34   | 2.2                            | 4.48  | 3.48   | 3.75   |
| Thyme   | 4.99              | 4.73  | 3.24   | 3.85   | 2.51                           | 5.08  | 3.4    | 3.94   |

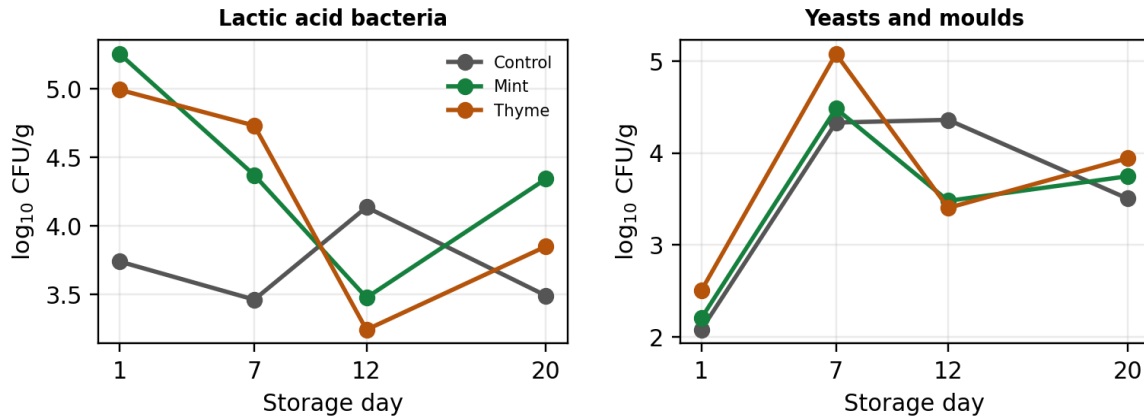
Source: Authors' research

In mint and thyme cheese, LAB declined progressively to day 12, reaching 3.48 and 3.24 log<sub>10</sub> CFU/g, respectively, before increasing to 4.34 and 3.85 log<sub>10</sub> CFU/g on day 20. The control fluctuated, with a maximum of 4.14 log<sub>10</sub> CFU/g on day 12 and a decline to 3.49 log<sub>10</sub> CFU/g on day 20. Such non-linear behaviour is plausible in fresh-cheese ecosystems, where acidification, nutrient depletion, salt, oxygen transfer and interspecies competition successively select different populations (Irlinger and Mounier, 2009; Settanni and Moschetti, 2010). The comparatively high final LAB count in mint cheese indicates persistence of viable LAB despite the plant additive, but species-level identification would be needed to distinguish technologically beneficial LAB from adventitious populations.

Yeasts and moulds displayed a pronounced rise by day 7 in every treatment. The highest value occurred in thyme cheese ( $120,000 \pm 42,426$  CFU/g;  $5.08 \log_{10}$  CFU/g), compared with  $30,500 \pm 1,556$  CFU/g in mint cheese and  $21,500 \pm 3,536$  CFU/g in the control. Counts subsequently decreased, but viable populations remained present on day 20 (3.51–3.94 log<sub>10</sub> CFU/g). Thus, neither herb consistently prevented fungal proliferation. Antimicrobial activity demonstrated for essential oils or extracts may be weakened when whole herbs are incorporated into protein- and fat-rich foods because active compounds partition into the matrix and their effective aqueous-phase concentration decreases (Burt, 2004; Tajkarimi et al., 2010; Gyawali and Ibrahim,

2014). The day-7 peak may also reflect oxygen exposure and post-processing contamination, as many spoilage yeasts and moulds originate from the dairy environment (D'Amico, 2014; Garnier et al., 2017). Although national legislation does not prescribe a general yeast-and-mould limit for this exact cheese category, the observed peak—especially in thyme cheese—signals reduced microbiological stability and should be considered when establishing shelf life.

**Figure 1. Changes in LAB and yeasts and moulds during 20-day storage.**



Source: Authors' research

The safety- and hygiene-related results are presented in Table 2. *E. coli*, anaerobic bacteria and *Salmonella* spp. were not detected in any cheese at any sampling point. Enterobacteriaceae were present only during early storage: 10 CFU/g in the control on day 7, 100 ± 14 CFU/g in mint cheese on day 1 followed by 10 CFU/g on day 7, and 30 CFU/g in thyme cheese on day 1. Their disappearance by day 12 is consistent with progressive acidification and microbial competition, although the low early counts still emphasize the importance of raw-material and process hygiene.

**Table 2. Hygiene indicators and selected microorganisms (CFU/g or qualitative result). ND: not detected.**

| Variant | Microorganism          | Day 1  | Day 7 | Day 12 | Day 20 |
|---------|------------------------|--------|-------|--------|--------|
| Control | <i>E.coli</i>          | 0      | 0     | 0      | 0      |
|         | <i>S.aureus</i>        | 0      | 0     | 10     | 0      |
|         | <i>B.cereus</i>        | 0      | 0     | 0      | 0      |
|         | Enterobacteriaceae     | 0      | 10    | 0      | 0      |
|         | <i>Salmonella</i> spp. | ND     | ND    | ND     | ND     |
| Mint    | <i>E.coli</i>          | 0      | 0     | 0      | 0      |
|         | <i>S.aureus</i>        | 0      | 0     | 0      | 0      |
|         | <i>B.cereus</i>        | 40     | 0     | 0      | 0      |
|         | Enterobacteriaceae     | 100±14 | 10    | 0      | 0      |
|         | <i>Salmonella</i> spp. | ND     | ND    | ND     | ND     |
| Thyme   | <i>E.coli</i>          | 0      | 0     | 0      | 0      |
|         | <i>S.aureus</i>        | 0      | 0     | 50     | 0      |
|         | <i>B.cereus</i>        | 0      | 0     | 0      | 0      |
|         | Enterobacteriaceae     | 30     | 0     | 0      | 0      |
|         | <i>Salmonella</i> spp. | ND     | ND    | ND     | ND     |

Source: Authors' research

*S. aureus* occurred sporadically at low levels: 10 CFU/g in the control and 50 CFU/g in thyme cheese on day 12, with no detection at the following sampling. *B. cereus* was found only in mint cheese on day 1 (40 CFU/g). These isolated low counts did not persist, suggesting that storage conditions and the developing cheese ecosystem did not favor their multiplication. Nevertheless, detection at any point supports continued control of milk quality, equipment sanitation, handler hygiene and refrigeration.

This favorable profile contrasts with recent retail findings for traditional raw-milk cheese, in which coagulase-positive staphylococci were frequently detected and some samples exceeded 10<sup>5</sup> CFU/g, confirming that low counts cannot be assumed without verified hygienic control (Antoszevska et al., 2024).

### 3.1. Regulatory assessment of microbiological safety

The national Rulebook classifies *Salmonella* as a food-safety criterion for cheeses made from raw milk or milk subjected to a lower heat treatment than pasteurization. The criterion requires absence in 25 g, with a five-unit sampling plan ( $n = 5$ ,  $c = 0$ ). *Salmonella* spp. was not detected in any analysed variant or storage interval; consequently, all reported analytical results were consistent with the required limit. Because only duplicate laboratory determinations from a single production batch were available, however, this dataset does not reproduce the statutory  $n = 5$  sampling plan and cannot independently support a formal declaration of conformity for commercial release. Comparable official-control data from 1,373 raw- and heat-treated-milk cheeses showed high overall conformity with food-safety criteria but lower conformity with process-hygiene criteria, reinforcing the need to distinguish product safety from manufacturing hygiene when interpreting cheese results (Primavilla et al., 2023).

For cheese made from raw milk, coagulase-positive staphylococci are evaluated as a process-hygiene criterion, with  $n = 5$ ,  $c = 2$ ,  $m = 10,000$  CFU/g and  $M = 100,000$  CFU/g at the stage when the highest count is expected. The maximum observed *S. aureus* value was 50 CFU/g, which is 200 times lower than  $m$  and 2,000 times lower than  $M$ . On a numerical basis, all three variants therefore satisfied this safety-relevant hygiene benchmark. The result is reassuring, but it should be described as compliance of the measured values rather than full legal batch conformity because the required number of sample units was not examined.

*E. coli* and Enterobacteriaceae were interpreted as supplementary indicators of faecal or process hygiene. The current product-specific criteria do not establish a general limit for these organisms in raw-milk fresh cheese equivalent to the criteria for certain heat-treated cheeses. Their absence or low early counts therefore support satisfactory hygiene but do not, by themselves, determine legal acceptance. Likewise, the Rulebook does not set a general product-specific safety limit for LAB, yeasts and moulds, *B. cereus* or total anaerobes in this cheese category. LAB counts primarily describe fermentation ecology, whereas yeasts and moulds are more directly relevant to spoilage and shelf-life validation. A high fungal count can be technologically unacceptable even when it does not constitute non-compliance with a specified food-safety criterion.

**Table 3. Regulatory interpretation of the microbiological results under the national criteria.**

| Parameter                               | Applicable criterion                                    | Observed maximum        | Assessment                            | Interpretive status                                            |
|-----------------------------------------|---------------------------------------------------------|-------------------------|---------------------------------------|----------------------------------------------------------------|
| <b>Salmonella spp.</b>                  | Absent in 25 g; $n=5$ , $c=0$                           | Not detected            | Within limit                          | Food-safety criterion; indicative because $n=5$ was not tested |
| <b>Coagulase-positive staphylococci</b> | $m=10,000$ , $M=100,000$ CFU/g; $n=5$ , $c=2$           | 50 CFU/g                | Within limit                          | Process-hygiene criterion; measured values comply              |
| <b>E. coli / Enterobacteriaceae</b>     | No general product-specific limit for this category     | 0 / 100 CFU/g           | Favourable hygiene profile            | Supplementary indicators                                       |
| <b>B. cereus / anaerobes</b>            | No general product-specific criterion for this category | 40 / 0 CFU/g            | No safety-limit exceedance identified | Supplementary monitoring                                       |
| <b>LAB / yeasts and moulds</b>          | No statutory safety limit for this category             | 180,000 / 120,000 CFU/g | Shelf-life interpretation required    | Technological and spoilage indicators                          |

Source: Authors' research

Overall, the herb-enriched cheeses differed from the control in their initial LAB load and in the magnitude of the day-7 yeast and mould peak. However, the direction of change was not uniformly antimicrobial: thyme cheese had the highest fungal count on day 7, while mint cheese retained the highest LAB count on day 20. Whole

herbs may introduce their own microbiota, and the bioavailability of antimicrobial compounds may be reduced by binding or partitioning within the cheese matrix. Future validation should include at least three independent production batches, the legally required number of sample units, a defined herb dose, pH and water activity at every sampling point, confirmed refrigerated storage conditions and species-level identification of dominant LAB, yeasts and moulds.

#### 4. CONCLUSION

Mint and thyme altered the microbiological succession of fresh cheese during 20 days of storage, but neither herb produced consistent broad-spectrum inhibition. Herb-enriched cheeses had higher initial LAB counts than the control, followed by a pronounced decrease through day 12 and partial recovery by day 20. Yeasts and moulds increased sharply on day 7 in all variants, most notably in thyme cheese, and remained detectable at the end of storage. *E. coli*, anaerobes and *Salmonella* spp. were not detected, while *S. aureus*, *B. cereus* and Enterobacteriaceae appeared only sporadically at low levels. The absence of *Salmonella* and the very low maximum *S. aureus* count were consistent with the applicable national limits. Nevertheless, formal legal conformity cannot be claimed from analytical duplicates of one batch because the prescribed sampling plan was not fully implemented. The products showed a favorable safety profile, while the fungal dynamics indicate that shelf-life control and refrigerated storage remain essential.

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