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# MICROBIOLOGICAL QUALITY OF SLOVAK TRADITIONAL CHEESE

**Cabanová, L., Niníková, P.**

Veterinary and Food Institute
Jánoškova 1611/58, 02601 Dolný Kubín,
Slovakia

lenka.cabanova@svpu.sk

## ABSTRACT

**The aim of this study was to investigate the microbiological quality of traditional Slovak "bryndza" cheese made in Slovakia. Besides the common pathogenic bacteria, we focused on the analyses of verocytotoxigenic *Escherichia coli* (VTEC), the occurrence of which has been analysed only occasionally in a few products. As we chose food of the highest risk which contained raw milk, we expected several positive findings. The presence of *Salmonella* spp., *Listeria monocytogenes* and *Campylobacter* spp. was not confirmed. The enumeration of *Staphylococcus aureus* was more successful. In the case of VTEC stx and eae screening, the presence of genes producing verocytotoxins vtx1, vtx2 and the gene encoding virulent factor intimin—eae in nine samples by molecular-biological methods were revealed. Only one isolate, which carried genes vtx1 a vtx2 and did not belong to these serogroups: O157, O111, O26, O103, O145, or O104, was detected by confirmation assays.**

**Key words: microbiology; pathogenic bacteria; raw milk; traditional Slovak dairy products**

## INTRODUCTION

In Slovakia, "bryndza" belongs to one of the traditional products the consumption of which is common and profitable from different points of view. Many scientific studies [1, 3, 19] state its positive influence because of the original microflora the composition of which is unique. After the proper technological procedures, "bryndza" flora can even act against many pathogenic bacteria [4]. It belongs to the much-sought-for favourite products and as a traditional food, it is prepared for tourists, mainly in the Slovak regions of Liptov and Orava.

However, based on its composition (it is made either from sheep only products—100 % sheep unpasteurized cheese, or from raw sheep cheese and pasteurized cow cheese 1 : 1), it can be considered as one of the foods with the highest risk. For this reason it has been advised that

pregnant women, small children or immunosuppressive patients avoid these kinds of products [11].

The Slovak State Veterinary and Food Administration and other related bodies (district organisations, veterinary and food institutes) pay special attention to the official control of this traditional product. Every year many samples taken from the producers or from retail outlets are analysed to decrease the risk to the consumers who buy the food that poses potential risk. Even the producers themselves pay attention to guarantee the safety of their products within their own controls.

The aim of this study was to investigate the microbiological quality of traditional Slovak "bryndza" cheese made in Slovakia. In addition to the common pathogenic bacteria, we focused on verocytotoxigenic *Escherichia coli* (VTEC), the occurrence of which has been analysed only occasionally in a few products.

## MATERIALS AND METHODS

In our study we analysed the "bryndza" cheese produced from either untreated sheep cheese (21 samples) or the mixture (1 : 1) of raw sheep and cow cheese (4 samples). All samples were analysed in accordance with the legislative requirements [6] and were made only by the Slovak producers from different geographical areas (Fig. 1). Three samples were taken at the retail level, and the remaining ones were provided by the producers. Every producer provided only one sample per production so that the variability of product types were as wide as possible. The samples did not reflect the total amount of production.

The samples were analysed for the presence of pathogenic bacteria, such as *Salmonella* spp. [17], *Listeria monocytogenes* [16] and coagulase positive staphylococci (CPS) [12] and only three samples were analysed for the occurrence of *Campylobacter jejuni* [15]. In the case of exceeding a count of $10^5$ CFU.g$^{-1}$ for CPS, staphylococcal enterotoxin was detected (using the VIDAS equipment). All samples were analysed in parallel for the presence of verotoxigenic *Escherichia coli* [14]. Similar results from these kinds of samples have not yet been obtained in Slovakia because the official controls of bryndza were conducted only occasionally in relation to human cases.

If negative results were obtained at screening, the procedure was terminated. In the case of positive findings, the pooled colonies [22] were further analysed by conventional PCR and the serotypes were identified.

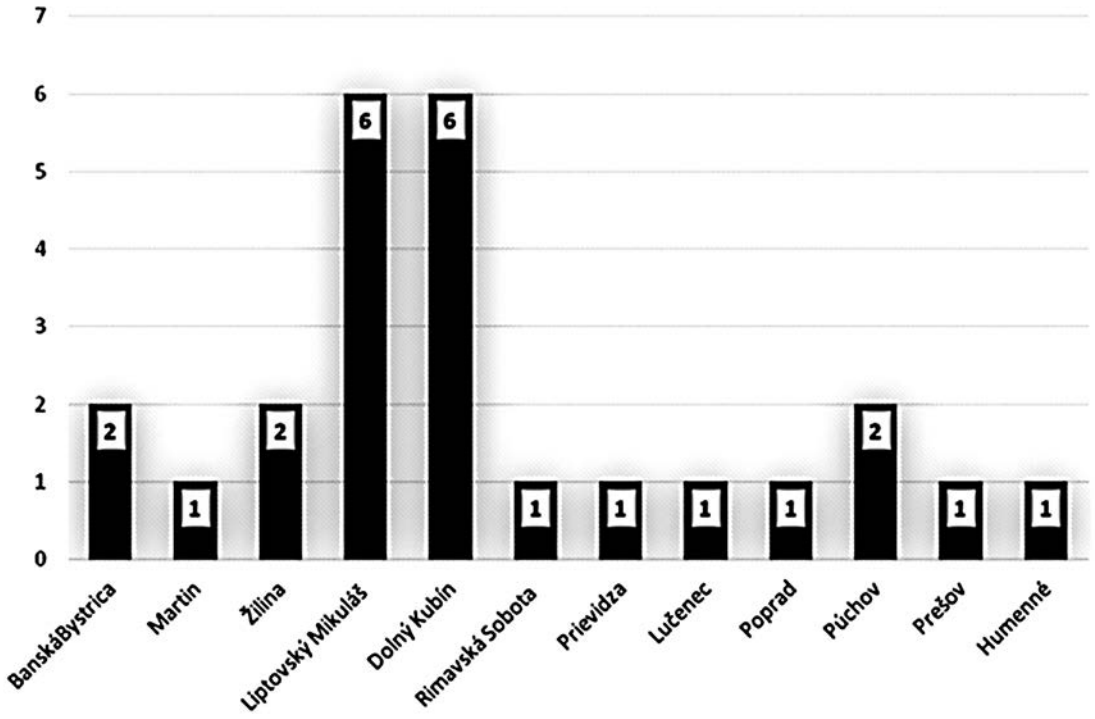


**Fig. 1. Number of producers involved according to the regional distribution**

The methodological procedure for the detection of the presence of verocytotoxin-producing *E. coli* (VTEC) was based on CEN ISO/TS 13136:2012 [14] and consisted of several steps. At first, incubation of the test portion was performed in a non-selective liquid nutrient medium—buffered peptone water (BPW) at 37 ± 1 °C for 18—24 hours. In the second step, 1 ml of microbial enrichment was transferred to an Eppendorf tube for isolation of the DNA [13]. The isolated DNA was subjected to a real-time PCR assay for the detection of virulence genes vtx1, vtx2 and eae in screening. The real-time PCR reaction was performed using Taq Man Universal PCR Master Mix (Applied Biosystems, Warrington, UK) in a thermocycler 7500 Real-time PCR System (Life Technologies, Applied Biosystems, USA). The results of the screening determined the next step of examination. The analyses of the negative samples with an absence of vtx genes were stopped. In the case of the presumptive detection of VTEC, the samples were subjected to the procedure for the determination of genes associated with serogroups. The positive colonies were analysed from pools by conventional PCR reaction [22] and then the serotype was characterised using real-time PCR.

## RESULTS

The absence of pathogenic bacteria indicated a high quality of the traditional unpasteurized products. Pathogenic bacteria (*Salmonella* spp. and *Listeria monocytogenes*) were not detected in any of the 25 samples. In the previous years, there was a monitoring programme conducted by the Veterinary and Food Institute (VFI) in Dolný Kubín that focused on *Campylobacter* spp. in the same kind of products [5]. It showed 3.41 % occurrence of *Campylobacter* spp. in unpasteurized dairy products. In our study, only three samples were analysed for *Campylobacter* spp. because the conditions to which the other samples were exposed were not suitable for this analysis. None of the 3 samples were positive for *Campylobacter* spp.

Based on the previous laboratory analyses of similar food matrix performed at our institute, high counts of CPS were expected. Increased levels were expected in correlation with summer temperature, poor storage conditions or problematic transport. Despite that, the results were better than expected (Tab. 1).

Cheese samples that according to the Commission Regulation 2073/2005 [6] exceeded the legal limit of 104 CFU.g-1 represented 4/25 (16 %). They were considered not to be in compliance with 2073/2005 and not suitable for human consumption. From these four samples there were two with counts exceeding 105 CFU.g-1. They were examined for the presence of staphylococcal enterotoxin and showed negative results.

The first screening of VTEC conducted in our study revealed the presumptive detection in nine samples (39 %) and also presumptive detection of enteropathogenic *E. coli* (EPEC) in three samples. EPEC generated the attaching and effacing lesions and carried the eae gene. Fig. 2 shows our results of VTEC screening.

The majority of the samples with the presumptive detection of VTEC belonged to a subset of enterohaemorrhagic *E. coli* (EHEC). This subset was characterized by the presence of vtx and eae genes and in this screening, six samples belonged to the EHEC group. In one case, the presumptive detection of vtx2 and eae was positive. In five samples the screening revealed the presence of both vero-

**Table 1. CPS counts detected in samples (CFU.g$^{-1}$)**

| Sample No. | Count | Sample No. | Count | Sample No. | Count | Sample No. | Count | Sample No. | Count |
|---|---|---|---|---|---|---|---|---|---|
| 1 | $1.4 \times 10^2$ | 6 | $1.0 \times 10^4$ | 11 | $< 1.0 \times 10^1$ | 16 | $1.3 \times 10^3$ | 21 | **$3.2 \times 10^5$** |
| 2 | $5.0 \times 10^3$ | 7 | $< 1.0 \times 10^1$ | 12 | $< 1.0 \times 10^1$ | 17 | $< 1.0 \times 10^1$ | 22 | $3.2 \times 10^2$ |
| 3 | $3.2 \times 10^2$ | 8 | $< 1.0 \times 10^1$ | 13 | $5.5 \times 10^2$ | 18 | $8.0 \times 10^3$ | 23 | $< 1.0 \times 10^1$ |
| 4 | $2.5 \times 10^2$ | 9. | $8.2 \times 10^3$ | 14. | **$1.5 \times 10^5$** | 19. | $3.0 \times 10^3$ | 24. | $3.0 \times 10^2$ |
| 5 | $< 1.0 \times 10^1$ | 10. | $4.0 \times 10^3$ | 15. | $8.0 \times 10^4$ | 20. | $8.2 \times 10^2$ | 25. | $9.1 \times 10^3$ |

Samples in bold were analysed for the presence of staphylococcal enterotoxins

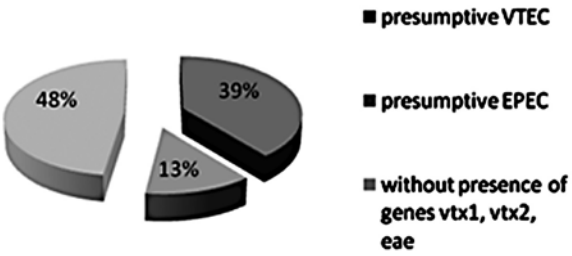


**Fig. 2. Results of PCR screening of cheese samples in liquid nutrient medium for VTEC detection (VTEC—erocytotoxigenic *E. coli*; EPEC —enteropathogenic *E. coli*)**

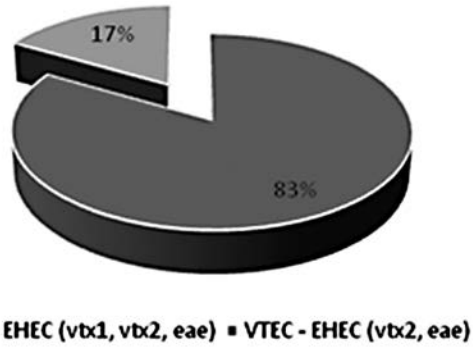


**Fig. 3. Percentage of identified genes of virulence in EHEC isolates, obtained from PCR screening of liquid nutrient medium with cheese samples for VTEC detection (VTEC-EHEC—verocytotoxigenic *E. coli* belonging to the subset of enterohaemorrhagic *E. coli*)**

cytotoxin genes vtx1 and vtx2 and intimin-coding gene eae (Fig. 3). These findings mean that in the test portion the presumptive detection indicated the presence of VTEC responsible for the attaching and effacing lesions on the gut mucosa.

The examination of pools from isolated colonies was used for the confirmation of the presumptive detection of genes vtx1, vtx2 and eae in cheese samples. These test assays were based on conventional PCR [22]. The incidence of vtx1 and vtx2 genes were confirmed in the pool made by the colonies. The eae gene encoding the virulent factor intimin was not detected. This is the usual finding. The pool consisted of ten colonies, but every colony could harbour different genes, combination of the genes, only one of them or none. This is the reason why we did not detect an isolate harbouring genes vtx1, vtx2 and also eae, but only the isolate harbouring genes vtx1 and vtx2. The isolated strain of *E. coli* underwent the identification of serogroups, but these did not belong to the highly pathogenic serogroups O157, O111, O26, O103, O145, or the serogroup O104.

## DISCUSSION

Cheese is generally considered a safe and nutritious food, but foodborne illnesses linked to cheese consumption have occurred in many countries. Many of these foodborne outbreaks were caused by the contamination with *Staphylococcus aureus. S. aureus* infections have been linked to the use of unpasteurized milk or to the contamination due to improper handling [8]. In our study we did not detect the staphylococcal enterotoxin. In the past 10 years there was only one sample of raw cheese positive for staphylococcal enterotoxin, type C.

The majority of the human outbreaks due to the consumption of raw milk cheeses were caused by *Salmonella* spp., followed by VTEC. The consumption of contaminated soft and semi-soft cheeses is often implicated in outbreaks with VTEC, especially when they are made from raw cow or goat milk [25]. In Slovakia, *Salmonella* spp. occurs usually in fresh poultry meat. In the dairy products investigated in 2014, two *Salmonella* serotypes were detected in positive samples: *Salmonella enterica* subsp. enterica serovar *Enteritidis* and *Salmonella enterica* subsp. enterica serovar *Montevideo*. These samples were of the same product type—traditional Slovak cheese made from raw sheep milk.

The most common pathogenic bacterium in Slovakia seems to be *Listeria monocytogenes*. This pathogen occurs not only in cheeses made from raw milk but also in those made from pasteurized milk (sheep, goat or cow, or in combination) as a consequence of failure in the pasteurization process [18]. This problem is linked to the handling and manipulation with the final product after heat treatment; although Ortenzi et al. [21] reported that *Listeria* counts decreased during the proper ripening and storage.

The most important results seem to be those obtained by the VTEC analysis. Baylis [2] mentioned that the prevalence of VTEC in products made from raw milk was very low. The outbreaks were caused mainly by enterotoxigenic *E. coli* (ETEC) and enteroinvasive *E. coli* (EIEC). However, VTEC was also responsible for some cases leading to the development of haemolytic uremic syndrome (HUS) in humans. The contamination of raw milk with VTEC is a significant problem not only for fresh cheese

curd, but also for hard ripened cheeses, because of the ability of VTEC to survive during the production procedure—periods of maturation. In 2002, during an outbreak in Canada, two strains of *E. coli* O157:H7 were isolated from unpasteurized Gouda cheese. One isolate was found in a 104 day old cheese [7].

Our results are comparable with those reported by authors from other countries. In Turkey, 2 % of Van herby cheese samples were positive for *E. coli* O157:H7 [24]. A study conducted in Italy revealed contamination in 7 % of the examined samples, but only three strains were detected. One isolate which originated from mozzarella cheese and two isolates from raw milk were positive for stx2 genes. Serotypes of these *E. coli* strains were not determined [20]. The results from Iran showed contamination of the traditional cheese by vtx2 and vtx1 in 120 (2.5 %) of the samples. In one of the cheese samples, the vtx1 gene was detected. Also, vtx2 gene was confirmed, in two of cheese samples [23]. According to EFSA data, thirty-six cheeses were positive for VTEC in 2016. Only one of these samples was E. coli O157 that originated from raw cow's milk. The majority of isolates belonged to the serogroup O26 [10]. Ten years ago, six *E. coli* O157 were found in cheeses. One strain was isolated in Slovakia from forty samples (2.5 %). Five Belgian strains were collected from soft and semi-soft cheeses made from unpasteurized cow's milk. Both of the countries tested samples only for the presence of serotype O157 [9] but no virulence genes were analysed at that time.

## CONCLUSIONS

The analysis of Slovak traditional bryndza cheese demonstrated the need for continuous monitoring of production procedures. Because of specific characteristics and the consumer's popularity of this cheese, systematic monitoring of its microbiological safety appears necessary. It is known that its composition and the original microflora show antimicrobial effects on the pathogenic bacteria. In our case, the most probable bacteria to survive in such a matrix seem to be coagulase-positive staphylococci.

With respect to VTEC results, they were the first obtained from the Slovak traditional food. Not only pathogenic *E. coli* were detected but also the presence of genes, associated with the production of verocytotoxins vtx1, vtx2 and eae, were investigated. In our study that included 25 samples, one VTEC isolate was detected (4 %). The screening revealed the presumptive detection of vtx1, vtx2 and eae genes in 56 % and the presumptive detection of vtx2 and eae genes in 11 %. From the point of view of food safety, this contamination would represent the highest risk to consumer's health. The contamination of cheeses by *E. coli* is not unique. Many findings have been described in Europe and also throughout the world. The practice of good manufacturing procedures is one of the most important measures in order to ensuring the production of safe foods. It is the right way to avoid or minimise the detrimental effects of pathogenic bacteria.

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