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# ASSESSMENT OF RAW MILK MICROBIAL QUALITY AND SURVEY OF THE OCCURRENCE AND EVALUATION OF THE PUBLIC HEALTH RISK OF *ESCHERICHIA COLI* IN RAW MILK FROM SMALL-SCALE INTEGRATED BACKYARD CATTLE FARMS IN WESTERN ROMANIA

## APRECIEREA CALITĂȚII IGIENICE ȘI EVALUAREA RISCULUI PENTRU SĂNĂTATEA PUBLICĂ A TULPINILOR DE *ESCHERICHIA COLI* DIN LAPTELE CRUD PROVENIT DIN FERMELE DE VACI DE LAPTE DE MICI DIMENSIUNI DIN VESTUL ROMÂNIEI

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**ABSTRACT | REZUMAT**

The survey was undertaken to investigate the microbial quality and the occurrence of *Escherichia coli* bacterial strains, as well as to evaluate their human infective potential, in a total of 54 raw milk samples collected from different small-scale integrated backyard cattle farms, located in five counties (Arad, Bihor, Caraș-Severin, Hunedoara, and Timiș) of Western Romania. A total of 51.9% (28/54) of the examined food matrices expressed higher total aerobic plate count (>5 $\log_{10}$ CFU/ml) than the one provided as the maximum admitted value by the EU legislation in force. *E. coli* was isolated from 24 (44.4%) samples. Results of molecular survey highlighted the presence of *stx2* gene in eight (33.3%) of the isolated *E. coli* strains, but none of the screened samples were positive for *elt*A, *est*I, *est*II and *stx*1 virulence genes, responsible for encoding of toxin synthesis. Our findings indicate the microbial quality of the raw milk in the screened area can represent a potential public health risk.

**Keywords**: raw milk, quality, *E. coli*, toxicity, gene

Studiul a fost realizat în vederea aprecierii calității microbiene a laptelui, incluzând detecția tulpinilor de *Escherichia coli* și pentru a evalua potențialul infecțios al acestora pentru consumatori. În acest scop au fost prelucrate un total de 54 de probe de lapte crud, recoltate din ferme de bovine de tip gospodăresc, de mici dimensiuni, situate în cinci județe din vestul României (Arad, Bihor, Caraș-Severin, Hunedoara și Timiș). Din probele de lapte examinate 51,9% (28/54) au prezentat valori crescute ale numărului total de germeni mezofili aerobi (NTGMA>5$\log_{10}$ UFC/ml), depășind valoarea maximă admisă de legislația Uniunii Europene în vigoare pentru acest indicator. *E. coli* a fost izolată din 24 (44,4%) de probe. Analizele moleculare au evidențiat prezența genei stx2, responsabilă de producția de toxine Shiga, la 8 (33,3%) din tulpinile izolate de *E. coli*, dar nici una dintre probele examinate nu a fost pozitivă pentru genele de virulență *elt*A, *est*I, *est*II și *stx*1, responsabile pentru codificarea sintezei de toxine. Rezultatele obținute reflectă un potențial risc pentru sănătatea publică a laptelui crud în zona luată în studiu.

**Cuvinte cheie**: lapte crud, calitate, *E. coli,* toxicitate, genă

The cow's milk is considered one of the most important animal origin foodstuffs used in human nutrition. However, the raw milk is considered an extremely perishable food matrices, having a high-water concentration (aw=0.99), together with important amount of nutrients (e.g., proteins, carbohydrates, lipids, mineral salts, and vitamins). These particularities generate a favourable environment for the development of contaminating adulteration and, sometimes, pathogenic microorganisms. In order to limit the public health risk associated with the consumption of insalubrious milk, the compliance of the hygiene rules established in EU regulations, both in the primary stage of the food chain, as well as during processing units, must to be considered a mandatory task (9, 15, 19, 20, 22).

*Escherichia coli* is a representative species of the genus *Escherichia*, taking part from the *Enterobacteriaceae* family. It is a faecal origin bacterium, having as habitat the digestive tract of humans and warm-blooded animals, and it is considered a sanitary hygiene indicator which reflect the faecal origin contamination of animal origin foodstuffs. However, even if *E. coli* is

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considered to be a faecal coliform epiphyte bacterium of the intestines of animals and humans, some strains can exhibit pathogenic mechanisms on the consumer, causing diarrheal syndrome. In these regards, within the pathogenic *E. coli* strains there are six sub-pathotypes including, enterotoxigenic (ETEC), enteropathogenic (EPEC),enteroaggregative (EAEC),enteroinvasive (EIEC), attaching and effacing (A/EEC) groups, as well as enterohemorrhagic (EHEC, also known as Shiga toxin-producing *E. coli* [STEC]) strains.

In the last period, a particular attention is paid for the consumption of animal origin foodstuffs providing from unpolluted areas. Another trend of the modern consumer is to consume foods in its natural state, with a slow and short heat treatment, in order to preserve its nutritional principles, vitamins, enzymes and other valuable compounds. In a recent review article and several other scientific papers conducted in the field of food safety was highlighted the fact that over the last decade, although the scientific achievements and progresses in the study of foodborne pathogens in Romania were remarkable, it is obvious that the diversity and number of scientific reports are limited, and additional studies focusing on the monitoring of the biological safety of food products are still required (5, 6, 16, 17).

The present study aimed to provide data on the overall assessment of the hygienic quality of the raw cow milk, collected from the extensively raised cow farms though the monitoring of two hygienic-sanitary indicators namely, the total aerobic plate count (TAPC) and *E. coli,* and molecular evidence of the virulence and toxicity genes exhibited by of the isolated *E. coli* strains to provide valuable data of the risk that is posed to the public health, associated with the consumption of raw milk and milk derived products (3, 4, 8).

## MATERIALS AND METHODS

Between October 2017 and April 2018, a total of 54 raw milk samples were collected directly from the cooling container, immediately after milking, of the small-scale integrated backyard cattle farms (up to 30 heads) located in five counties (Arad, Bihor, Caraș-Severin, Hunedoara, and Timiș) of Western Romania.

From each county, excepting Timiș, randomly selected ten primary production units were enrolled in the study, agreeing to participate in the survey.

The samples (~500 ml) were aseptically collected in sterile universal bottles, stored under refrigeration and transported (for a maximum of four hours) to the laboratory of Food Hygiene of the Faculty of Veterinary Medicine Timișoara, Romania.

All of the collected samples were processed on the day of collection for microbiological analysis.

For each raw milk sample, the TAPC determination was performed according to the SR EN ISO 4833-1: 2014, SR EN ISO 4833-2:2014, as has been previously described (7, 21).

For the isolation and determination of the numbers of *E. coli* strains, the horizontal method for counting *E. coli* (positive for β-glucuronidase) was used, following the steps described in the SR ISO 16649-2/2007.

The isolated colonies were molecularly confirmed by polymerase chain reaction (PCR), using the universal primer set and cycling conditions designed by Srivastava et al. (2008), and targeting the 16S rDNA gene. In addition, the isolated *E. coli* strains, in order to evaluate their human infective potential, were molecularly screened for the presence of several genes encoding the toxin synthesis. In this regard, the specific primer sets and annealing temperatures were used according to the correspondent references, as presented in the Table 1 (13, 21).

## RESULTS AND DISCUSSIONS

The study results are summary presented in Table 2. Out from the 54 processed raw cow milk samples, 26 (48.1) exhibited a relatively low overall microbial load, meaning values up to 100.000 (5 $\log_{10}$) colony forming

**Table 1**

**The targeted virulence genes and corresponding primer pairs and annealing conditions used**

| Gene | Toxin | The used primer sequences | Annealing temperature | Expected size of the amplified product | References |
|---|---|---|---|---|---|
| *stx*1 | STx1 | F: CGC TGA ATG TCATTC GCT CTG C<br>R: CGT GGTATA GCT ACT GTC ACC | 60 | 302 | 18 |
| *stx*2 | STx2 | F: CTT CGG TAT CCTATT CCC GG<br>R: CTG CTG TGA CAG TGA CAA AAC GC | 60 | 516 | |
| *elt*A | LT | F: ATT TAC GGC GTT ACT ATC CTC<br>R: TTT TGG TCT CGG TCA GAT ATG | 59 | 275 | 8 |
| *est*I | STa | F: TCC CCT CTT TTA GTC AGT CAA CTG<br>R: GCA CAG GCA GGATTA CAA CAA AGT | 60 | 163 | 4 |
| *est*II | STb | F: GCA ATA AGG TTG AGG TGAT<br>R: GCC TGC AGT GAG AAATGG AC | 60 | 368 | 3 |

**Table 2**

**Overview of the analysed raw milk hygienic quality and the occurrence of *Escherichia coli* strains, their contamination level and *stx2* gene positivity in the screened 54 small-scale integrated backyard cattle farms**

| County (no. of screened farms) | Total aerobic plate count ($log_{10}$ CFU/ml) | | *Escherichia coli* strains | |
|---|---|---|---|---|
| | Minimum | Maximum | No. of positive farms (minimum – maximum, $log_{10}$ CFU/ml) | *stx2* producing |
| Arad (10) | 4.176 | 5.290 | - | |
| Bihor (10) | 4.863 | 5.149 | 6 (2.255 – 3) | 2 |
| Caraș-Severin (10) | 1.982 | 5.913 | 4 (1 – 1.301) | 1 |
| Hunedoara (10) | 4.778 | 6.121 | 6 (2.602 – 3.751) | 2 |
| Timiș (14) | 3.954 | 5.896 | 8 (1.477– 3.342) | 3 |

units per millilitre (CFU/ml). Also, in 18 (33.3%) samples values between 5.004 $log_{10}$ and 5.699 $log_{10}$ CFU/ml were registered, and in other 10 (18.5) samples the recorded values were higher than 5.699 $log_{10}$ (CFU/ml). Quoting of the number of *E. coli* CFU was performed according to the developed typical blue-green colonies on Petri dishes (Fig. 1), as consequence of the penetration of chromophore substances, presents into the culture media, into the bacterial wall.

Thus, *E. coli* colonies were isolated in 24 (44.4%) samples. Six (11.1%) samples showed a very low (up to 2 $log_{10}$) contamination level, and in another six samples a low (up to 3 $log_{10}$) contamination level was observed. A high *E. coli* contamination level (> 3 $log_{10}$) of was found in 12 (22.2%) raw milk samples.

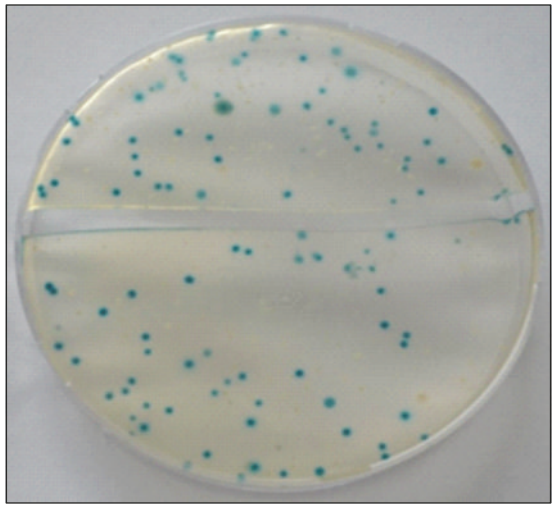

**Fig. 1.** Petri dish with typical blue-green colour of *E. coli* colonies

All of the isolated *E. coli* strains using non-molecular tools were successfully molecularly confirmed, showing characteristic bands at 810 base pair (Fig. 2).

Monitoring of the ETEC/VTEC virulence genes, encoding specific *E. coli* toxin synthesis, revealed the presence of the *stx2* gene (516 bp) in 8 (33.3%), and absence of the other four (*elt*A, *est*I, *est*II and *stx*1) screened genes in all of the processed samples.

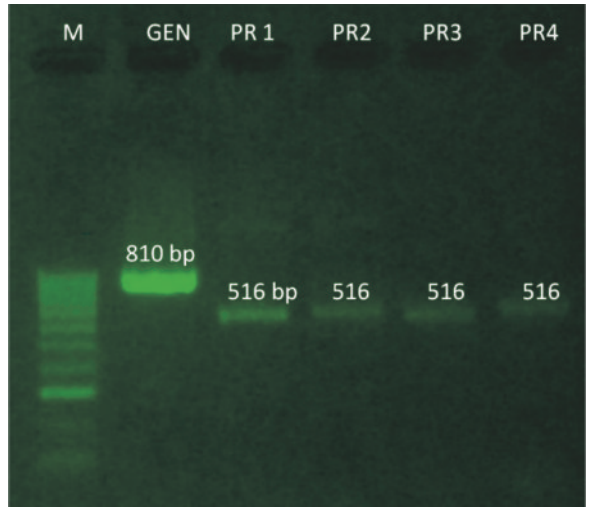


**Fig. 2**. Electrophoresis gel image indicating positive results; M - molecular marker, GEN - molecular confirmation of the presence of *E. coli* species based on 16S rDNA gene amplification; PR1-PR4 molecular evidence of the presence of the stx2 gene at 516 bp

The present study results demonstrated that the overall hygienic quality of the screened raw milk, providing from 54 small-scale integrated backyard cattle farms, situated in five western Romanian counties, only partially meets the requirement of the European legislation in force. Accordingly, EU Regulation No. 853/2004 provides a maximum limit for TAPC of 100.000 (5 $log_{10}$) CFU/ml raw cow milk, as a geometric mean for a period of two-month, with at least of two sampling per month. Although the primary production units were not have been monitored for at least two months, according to the EU requirements (i.e., four sampling and analysis for each farm), the exceeding of the maximum admitted level for the more than half (28/54; 51.85%) of the processed samples, can constitute a concern for the public health. The most important contamination sources for the raw milk are considered to be the skin and the mammary gland of the milking animals together with the unhygienic milking equipment. In a study conducted in order to investigate the influence of different contamination

sources on the hygienic quality of the raw milk, Alemu and Assefa (2016) demonstrated that a simple washing of the mammary gland with clean water leads to a reduction in the microbial load of the raw milk by more than 50% (1, 9, 22).

Similar to our findings, higher non-compliance values were observed by Sala et al. (2014) in a survey conducted in Timiș county, where 77.8% (95/122) of the processed raw milk samples expressed bacterial counts higher than 5 $\log_{10}$ CFU/ml.

Although the TNMAB indicator does not provide direct indications about the potential risk to the consumer, its significance is strongly related by the relationship of direct proportionality between the recorded nonconformity values and the probability of selecting pathogenic bacterial strains with significance for the public health. In this regard, special attention should be granted to dairy products obtained from raw milk (e.g., different cheese assortments) without thermal treatment. To clarify these aspects, further investigation, processing a representative number of samples from each farm, are recommended (12).

Concerning the recorded prevalence values of *E. coli* bacteria in the processed samples (44.4%), higher (63%) contamination levels has been reported by Ali and Abdelgadir (2011) in the Khartoum North state, Sudan, and lower (20.7%) by Adesiyun (1994) in the screened bovine milk samples in Trinidad. Even if *E. coli* is not considered to be a hygiene indicator for the raw milk, its presence and number suggests a recent faecal origin contamination. This bacterium is not resistant to the thermal treatments and environmental conditions, most strains being considered harmless, but some are recognized as causative agents for severe digestive and extra intestinal disorders (2, 5).

Results of molecular survey highlighted the presence of *stx2* and the absence of *elt*A, *est*I, *est*II, and *stx*1 ETEC/VTEC virulence genes of the screened *E. coli* strains, responsible for encoding of toxin synthesis. Similar to our study, the presence/absence of the aforementioned genes, aiming to evaluate the human infective potential of the isolated *E. coli* strains from milk and dairy products, have also been monitored in another surveys. Thus, in Romania, Tabaran et al. (2017), in a study with similar design to the current investigation and achieved in North-Western region of the country, obtained that 18.6% (27/145) of the isolated *E. coli* strains from the processed raw milk (n=120) and traditional dairy cheeses (n=80) marketed in Romania were found positive for one of the investigated toxicity genes. In this study, 44.4% of the isolated strains were found positive for *stx* genes, and none of the samples were positive for *est*I gene. However, 9.7% of the tested isolates harboured both *elt*A and *est*II genes, besides other 2% that showed positivity for both *stx*1 and *stx*2 genes.

In another study conducted in France, Vernozy-Rozand et al. (2005) highlighted the presence of the *E. coli stx*-positive strains in a significant number (n=32) of the tested cheese samples obtained from raw milk, highlighting the need of the implementation of effective pre- and post-operational control measures.

In addition, in a study aiming the isolation and molecular characterization of the virulence genes of *E. coli* strains from milk and milk products, Virpari et al. (2013), showed that out from the obtained 80 isolates, 25 (31.3%) were positive for the *stx* gene. Out of them, 7 (8.8%) were positive only for the *stx*I gene and 12 (15%) only for the *stx2* gene, while 6 (6.3%) isolates were positive for both (*stx1* and *stx2*) genes.

Moreover, in another survey, Paneto et al. (2007) investigated the presence of enterotoxigenic *E. coli* in raw milk and various assortments of Brazilian raw milk origin cheeses. Of the 50 isolated samples, three (6%) were *E. coli* verotoxigenic (VTEC) expressing the presence of the *stx*1 gene (10, 14, 18, 19, 20).

## CONCLUSIONS

The current study highlighted that the overall hygienic quality of the analysed raw milk samples, providing from the production level of the surveyed primary production units, only partially satisfies the requirements of the European legislation. The occurrence of the *E. coli* strains in less than half on the analysed samples, with a relatively low contamination level ranging from 1 $\log_{10}$ to 3 $\log_{10}$ cells/ml of milk indicate a relatively low faecal contamination level. The lack of the correlation between the TAPC values and the contamination degree of the analysed raw milk with *E. coli* suggests the existence of other pollution sources along the raw milk production chain. Furthermore, the occurrence of the *stx2* virulence gene in 8 of the analysed samples, which encode the synthesis of *Shiga* toxin, highlights the importance of the heat treatment for the raw milk destinated for human consumption. In this regard, the presence of the potential toxigenic *E. coli* strains can be considered a concern for the public health, especially in case of direct purchasing of dairy products (e.g., raw milk, sweet cream or various fresh cheese assortments) from local producers. Last but not least, the results of this study emphasize the importance of the implementation of Good Hygiene Prac-tices, especially at the primary production level of the food chain.

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