

OCCURRENCE OF *ENTEROCOCCUS* SPP. ISOLATED FROM THE MILK AND MILK PRODUCTS

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ABSTRACT

Enterococcus spp. is the most controversial group of lactic acid bacteria that have been for years ascribed with beneficial or detrimental role in food and feed. The aim of our study was to monitor the occurrence of *Enterococcus* spp. as the indicator of the contamination from collected samples of raw cow's milk, goat's colostrum and whey (n = 186). Cultures of enterococci were cultivated and purified and identified by the genus-specific and species-specific PCR method (n = 230). Among suspected isolates in total 222 isolates (96.5%) were identified as *Enterococcus* spp. The results were the same in all samples separately, more than 90% each of them were positive to *Enterococcus* spp. The results of counting the number of cultivated colonies showed that the largest number of enterococci is found in the samples of whey taken after the process of electro dialysis and the smallest in the native whey sample. From collected whey samples, 64 samples (90%) were PCR positive for enterococci species. Afterwards within the identification of several selected isolates that were identified, as *Enterococcus* spp. by the species-specific PCR method the most frequently presented in all of isolates was *Enterococcus faecalis*. Apparently the presence of enterococci was detected in all samples, but in amounts that aren't hazardous for human health. Although enterococci are opportunistic pathogens, it seems that they occur frequently in foods (especially fermented) in large numbers.

Keywords: enterococci; PCR; raw cow's milk; colostrum; whey

INTRODUCTION

Lactic acid bacteria (LAB) is a group of Gram-positive bacteria united by a constellation of morphological, metabolic and physiological characteristics. The group consists of around 20 genera producing lactic acid as the end product of carbohydrates fermentation (Salmien et al., 2004).

The genus *Enterococcus* is the most controversial and one of the largest group that belongs to LAB group. They are Gram-positive homofermentative cocci, occurring singly or in pairs and can be found in variety of habitats including humans and animals. From the taxonomic point of view enterococci have been reviewed several times and today genus consists of at least 53 species of which *Enterococcus faecalis* and *Enterococcus faecium* are the most important (Giraffa, 1999; Giraffa, 2003; Khan et al., 2010). These bacteria play important role in the food and feed fermentation and nowadays these strains are frequently used as probiotics. They are considered as a potential cholesterol-lowering agents, in treatment of gastrointestinal diseases and for immune regulation (Franz et al., 2011). With their ability to produce enterocins (class II of bacteriocins) *Enterococcus* strains can provide natural preservation of dairy products and hurdle in the growth of microorganisms (antimicrobial activity against spoilage or pathogenic bacteria such as *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium* spp.). Also on the other hand enterococci have their negative influence – opportune pathogens and can cause diseases

(like endocarditis, bacteremia, infection of urinary tract, central nervous system, intra-abdominal and pelvic infections) and also carrying enzymes involved in biogenic amines production (Fouquié Moreno et al., 2006; Franz et al., 2011).

During the process of lactation colostrum is the first milk that comes from the mammary glands of mammals and after that raw milk. The consumption of colostrum for ruminant species (such as cow, goat and sheep), but also humans and other mammals has a fundamental role in passive immune transfer and in the survival rate of newborns. For that reason it is necessary that the newborns obtain adequate amount of colostrum during their first days of life to obtain adequate passive immune transfer and increase its future productivity. The amount and composition of produced colostrum can be affected by several factors such as nutrition or litter size (Uruakpa et al., 2002; Hernández-Castellano et al., 2015).

Milk is the most completed natural fluid, one of the most important basic and healthiest raw materials, which plays important role in the dairy nutrition of all population (necessary for human health and normal function of the human body). With its consumption we start pretty early (in early childhood, when we are born) and continue in all stages of life till the late elderly (Zulueta et al., 2009; Tratnik and Božanić, 2012). Goat's milk with its ameliorative composition of its components plays an important role in the specific diet and the economy of many developing countries (Guo, 2003).

During the manufacturing of milk after precipitation of casein the greenish translucent liquid that is obtained is whey. For a long time it has been viewed as one of the major disposal problems of the dairy industry, but not anymore. Today there are several types of whey, which depending mainly on the processing sequence resulting in casein removal from fluid milk, but the most often encountered types (sweet or acid whey) originates from the manufacture of cheese. With its nutritional aspects and enriched with some other additions like fruits is one of the healthy products that can be found on the market (Jelen, 2002, Pescuma et al., 2008; Tratnik and Božanić, 2012).

With its very complex and changeable composition, milk and dairy products are favorable environment for growing initial and subsequent microflora involved in fermentation or food spoilage.

The aim of this study was to monitor the occurrence of *Enterococcus* spp. in collected samples of raw cow's milk, goat's colostrum and whey as the indicators of contamination.

MATERIAL AND METHODOLOGY

All together 186 samples of raw cow's milk, goat's colostrum and whey were collected in the different areas of South Moravia Region in the Czech Republic in a period of 2013 and 2014. The samples of raw cow's milk were collected from milk vending machines available and goat's colostrum samples were collected from several dairy farms. Whey samples were only periodically collected in different manufacturing stages, from one dairy plant in South Moravia in the Czech Republic, in the period of middle of February to the middle of March 2014 (one month). The collection of samples and their origin are described in the Table 1.

Basic processing of the samples was carried out immediately according to the ISO 7218 and ISO 6887-1 standards. The amount of 25 mL of each sample was diluted in 225 mL of Buffered Peptone Water (Oxoid, England) and made decimal dilutions. From diluted sample

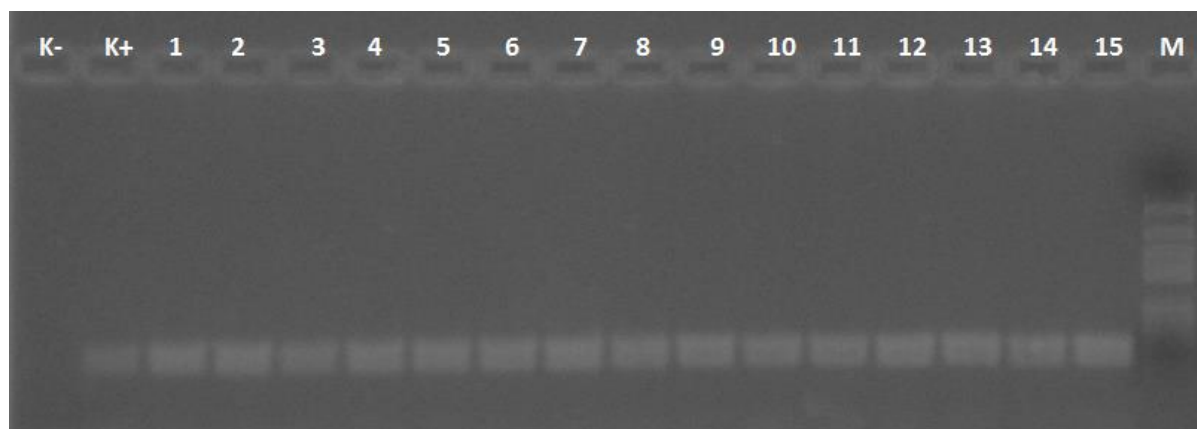
200 µL was aseptically spread on Slanetz Bartley agar (Oxoid) and cultivated at 37°C for 24h aerobically. All colonies from each sample that showed different morphological characteristics and pigment production were selected and purified for further characterization on Bile Esculin agar (Oxoid) and Blood agar (Oxoid).

Isolation of bacterial DNA was performed by 20% Chelex 100 (Bio-Rad Laboratories, USA). Isolates were identified by the genus-specific polymerase chain reaction (PCR) method based on the detection of *sodA* genes encoding enzyme mangan-dependent superoxide dismutase for the rapid identification of enterococci (Jackson et al., 2004). The PCR amplification was carried out in the PTC-200 thermocycler (MJ Research, USA). Isolates were also identified by the species-specific PCR method also according to the Jackson et al. (2004).

RESULTS AND DISCUSSION

A total amount of grown colonies, that after cultivation showed typical growth as enterococci was 230 suspected isolates (Table 1). Nowadays several molecular methods are available for identification of microorganisms. The commonly used molecular method for identification of enterococci is PCR method (Figure 1). Based on the detection of *sodA* genes encoding enzyme mangan-dependent superoxide dismutase for the rapid identification of enterococci, only 8 strains (3.5%) of suspected isolates didn't show the specific PCR product.

Enterococci occur as nonstarter LAB in variety of cheese production and ripening (e.g. the development of flavor) probably due to their thermal resistance during pasteurization of milk. High level of contaminating cheese with enterococci, except as starter cultures, can result in the poor hygienic practices during manufacturing (Pieniz et al., 2014). During all phases of whey manufacturing enterococci were presented (Table 2). This could be due to their ability of biofilm formation (electrodialysis) or maybe due to their resistance so they continually remain during the process. Whey is a by-product of the cheese



K-: negative control (reaction admixture with PCR H₂O), K+: positive control (*Enterococcus* spp., 112 bp), M: marker (155-970 bp), 1-15: *Enterococcus* spp. (112 bp)

Figure 1 Agarose gel electrophoresis of PCR products with genus-specific primers obtained by DNA amplification of 15 bacterial strains of the genus *Enterococcus*.

industry, which was often disposed as the waste. During the cheese production some compounds present in milk and / or cheese end up in the composition of whey. The major factors to determine the survival of this bacteria include particular characteristics of the strains, composition of food ingested and competition of microbiota (Pieniz et al., 2014). Whey and its protein content are ingredients used in dairy industry mainly due to their foaming and emulsifying properties and increasing nutritional quality (cheese, dairy desserts etc.). LAB in whey can decrease the high content of lactose and increase the digestibility of BLG (β -lactoglobulin) and ALA (α -lactalbumin) proteins. This can contribute to the change and increase the flavor aroma and texture of the end product and provide extra nutritional value (Pescuma et al., 2008). Enterococci were present in all phases of whey processing, except in the native way sample, but in the amount that is not hazardous for human health. Besides of maybe bad hygienic conditions, handling with and cleaning the equipment during whey manufacturing, the high prevalence of enterococci in whey may be also attributed to their resistance to heat, acid, salt and harsh conditions during food processing. To conclude, the application of electro dialysis, in the process of whey manufacturing, proved to be good.

Today, the consumers are offered a wide range of commercial types of milk including raw milk sold by milk vending machines or on the farm. According to the Food and Agriculture Organization of the United Nations (FAO)

that number is daily increasing. Not like before, within the consumption of raw milk consumers are today very concern about their health and what kind of food they are consuming.

The difference in microbiological quality of raw milk collected out of vending machines at different places was noted. Within the microbiology analysis form collected samples of raw milk ($n = 30$) in total more than 90% of the collected samples ($n = 28$) contained *Enterococcus* spp. A total of 38 suspected enterococci isolates were obtained from collected samples of raw milk. During the cultivation period from each sample was selected one or more colonies, that showed different morphological characteristics. Analyzing the isolated DNA with the PCR method for the identification of *Enterococcus* spp. more than 90% of isolates showed positive results ($n = 36$) and only 2 of suspected isolates were identified as non-enterococci. The presence and detection of enterococci in the samples of raw milk during the collection period time has been showed repeatedly. Comparing to the other works made on the occurrence of enterococci in raw milk, results were similar (Kagli et al. 2007; Franciosi et al., 2009).

The major enterococci in raw milk originates from plants (silage), udder skin, storage tanks, where they come from environments contaminated by human and animal fecal material (water, soil, urban sewage etc.) cause they are already naturally presented in the intestinal microflora (Giraffa, 2003; Kagli et al. 2007).

Table 1 The origin and frequency of *Enterococcus* isolates in all samples.

Commodity	Number of samples	Number of positive samples	Number of suspected isolates	Number of positive isolates
raw cow's milk	30	28 (93.3%)	38	36 (94.7%)
goat's colostrum	85	82 (96.5%)	106	103 (97.2%)
whey	71	64 (90%)	86	83 (96.5%)
Total	186	174	230	222

Table 2 The origin and frequency of *Enterococcus* isolates in the samples of whey.

Commodity*	Number of samples	Number of positive samples	Number of suspected isolates	Number of positive isolates
native whey sample	6	0 (0%)	2	0 (0%)
w in drying station	6	5 (83%)	7	6 (86%)
w from the tank	9	9 (100%)	11	10 (91%)
w before the electro dialysis	13	13 (100%)	18	17 (95%)
w after the electro dialysis	13	13 (100%)	17	17 (100%)
desalted w before concentration	7	7 (100%)	13	13 (100%)
desalted w behind the pasteur	5	5 (100%)	8	8 (100%)
concentrated, desalted w	4	4 (100%)	5	5 (100%)
sample of dried powder	5	5 (100%)	5	5 (100%)
Total	71	64 (90%)	86	83 (96.5%)

*w = the sample of whey

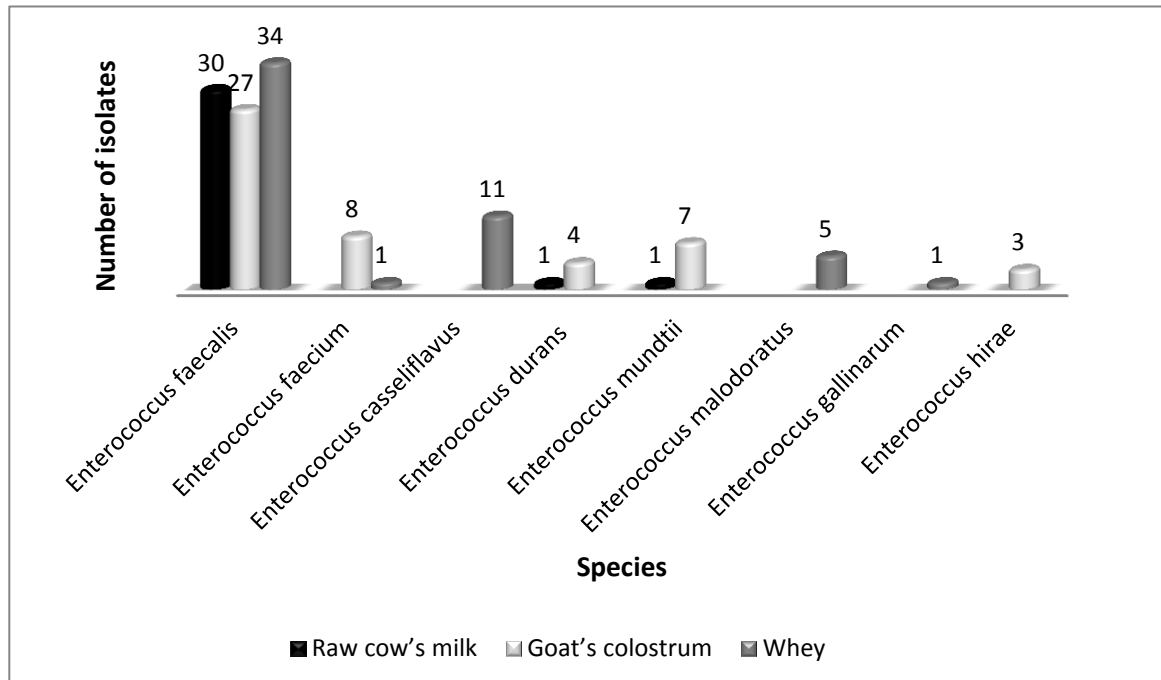


Figure 2 The frequency of several selected positive *Enterococcus* spp. Isolates.

For years it has been considered the milk is directly contaminated with fecal, but it seems that maybe due to the uncleaned milk equipment, milk vending machines and the lack of maintenance of hygienic conditions milk is becoming its source. Besides of that because of their psychrotrophic nature, their heat resistance and growth conditions, they can also increase during the milk refrigeration period (Giraffa, 2003).

Several of suspected isolates, which showed positive specific PCR product, were selected and subjected to further analysis. From each sample were taken several positive isolates and identified by the species-specific PCR method (Figure 2). The most of tested isolates were identified as *Enterococcus faecalis*, which is one of the most common species presented in the milk. According to the work of Citak et al. (2006) *Enterococcus faecalis* was detected in more than 50% of the isolated strains. Besides the *Ent. faecalis* and *Ent. faecium*, afterwards the major presence of *Enterococcus durans* was also identified as the most frequently presented (Kagli et al. 2007; Franciosi et al., 2009) and in our case especially in the samples of goat's colostrum.

CONCLUSION

This study confirmed the presence of *Enterococcus* spp. in all of collected samples: raw cow's milk, goat's colostrum and during the whole whey manufacturing process. Apparently the presence of enterococci in raw milk trails all the way from the colostrum since it is the first milk or from unkeeping the safty hygienic conditions. In whey manufacturing process the presented amount of enterococci is amout that is not hazardous for human health. Although enterococci are not pathogenic microorganisms, it seems that they occur frequently in foods (especially fermented) in large numbers. On the other hand besides the small share with their ability to

have beneficial effects on human health, these bacteria are accompanied with the great future opened for the long and more detailed exploration. Isolation of bacteria may have the advantage of giving the choice of safety for further operation in making dairy products.

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