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Georgian traditional soft cheese screening on existence of antibiotic resistant *Escherichia coli* and Coagulase-positive *Staphylococci* strains in Tbilisi, Georgia

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A B S T R A C T

The research aims to fill the knowledge gap of antimicrobial resistance spread through the Georgian dairy food chain and support countries' sustainable development in facing the global threat. Forty-two traditional Georgian soft cheese samples were collected from major organized retail and open markets in the Tbilisi capital of Georgia. All samples were tested on the presence of food pathogens *Escherichia coli (E. coli)* and Coagulase-positive *Staphylococci (CoPS)*, within limits set by the national regulation (№581:2015 and №301n:2001). Identified bacteria strains were tested on antimicrobial sensitivity for the top eight registered antibiotics used in veterinary.

Research showed alerting results, *E. coli* was detected in 92% of samples and *CoPS* in 42%. All strains of *E. coli* and *CoPS* show multidrug resistance toward different antibiotics.

Key words: Georgian Cheese, antimicrobial resistance, dairy sector, *E. coli*, *Staphylococcus aureus*, *Escherichia coli*, Coagulase-positive *Staphylococci*, food microbiology, food safety.

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Introduction

Safe food, free of pathogenic bacteria, is fundamental for food security. Providing nutrients necessary for body functioning and supporting sustainable development of United Nations (UN). Food security is endangered by the rapid development of antimicrobial resistance (AMR). Antimicrobial resistance is a silent pandemic threatening human health by endangering the ability to treat even modest infectious diseases [1]. Antibiotics in veterinary exceed usage in the health sector and are often misused [2]. However, more than 84% of countries do not have suitable legislation for veterinary drug control[3]. The deviation between human health and animal agriculture remains the main challenge that requires multidisciplinary work of all sectors and global pastorship as part of the One Health Approach [4]. AMR development in pathogens causes food poisoning outbreaks[5] and Antibiotic-resistant bacteria spread through the food chain.

Combating antimicrobial resistance is essential for the attainment of the Sustainable Development Goals (SDG; United Nations) and relates closely to accomplishing multiple SDGs (Fig. 1) [6]–[8]. Since 2015 Georgia adopted Sustainable Development Goals and initiated a strategy to combat AMR [9].

Fig. 1. *AMR reflection in SDGs*

Research question. This research aims to fill the knowledge gap of antimicrobial resistance spread in food pathogenic bacteria in the dairy industry, on the example of Georgian traditional soft cheese food chain. The research aims to supports countries' sustainable development road map, as according to National Strategy for Combating Antimicrobial Resistance, data concerning antimicrobial resistance spread through the food chain is deficient [10].

To address emerging risks, we have selected common food pathogens *Escherichia coli* (E. coli) and Coagulase-positive *Staphylococci* (CoPS) as research objectives. Collect cheese from organized retail and open markets located in Tbilisi capital of Georgia. Analyze the presence of pathogenic bacteria within the regulation limits, and assess antimicrobial sensitivity of identified strains, toward the top eight antibiotics registered for cattle veterinary [11] [12].

***Escherichia coli* and Coagulase-positive *Staphylococci* in food microbiology regulations.** *Escherichia coli* and Coagulase-positive *Staphylococci* are considered as hygiene indicators of the food industry internationally [11]. Indicating poor production hygiene, process failure, or inadequate process (e.g., pasteurization) or post-process contamination of food [13].

E. coli is spread in the food industry due to fecal contamination of raw materials. Usage of *E. coli* as a hygiene indicator is affected by the low cost of analyses and the possibility to receive results within 48hr, compared to five to seven-day long analyses necessary to identify other pathogens such as *Salmonella* [14]. Besides several types of *E. coli* are pathogenic by themselves, such as *E. coli* O157: H7, which has caused food poisoning outbreaks in different countries[14], [15].

Around 30% of the population is a carrier of CoPS [16]. CoPS causes different community enquired infections and produces heat-stable enterotoxins, which cause food poisoning[15]. Multidrug-resistant *Staphylococci* is presenting a global threat to human health [17]. Both *E. coli* and CoPS are the common causes of bovine mastitis. Recent studies suggest that methicillin-resistant *Staphylococcus aureus* (MRSA) strain, which causes more deaths than homicide, AIDS/HIV, and Parkinson's disease, originated from dairy cattle farms, transmitting through the food chain from cattle to farm workers[17][18].

Two legal acts regulate food microbiology in Georgia: Order of the Minister of Georgia №301/n "On Approval of Sanitary Rules and Norms on the Quality and Safety of raw food ingredients and Foodstuffs" and Resolution №581 "On the approval of the technical regulation on microbiological criteria for food"[19].

Each regulation sets acceptable limits for food microbiological criteria. №581 limits the number of *E. Coli* up to 1000 cfu/g in Cheeses made from milk that has undergone heat treatment, while №301/n prohibits coliforms, the working group of *Enterobacteriaceae*, including *E. coli*, in 0.001 g/cm^3 of cheese sample. Both regulations control fecal contamination risk. Limits for bacteria in the genus of *Staphylococci* also differ. №301/n allows up to 1000cfu/g *S. aureus* in cheese made with bacterial culture and prohibits presence in 0.1g/cm^3 of homemade cheese. №581 controls the group of Coagulase-positive *Staphylococci* bacteria, including *S. aureus*, while limits vary according to cheese heat treatment methods from 10^5 CFU/g for raw milk, 1000CFU/g for low heat treatment, and 100 CFU/g for pasteurized.

The difference between the two active regulations presents challenges for food business operators, who must comply with both. The historical development of food microbiology limits explains the difference. For example, at the end of the 20th century, coliform was replaced with *E. coli* as an indicator of food industry fecal contamination due to the higher accuracy of *E.coli* analyses [14][20]. № 301/n was published in 2001 using Soviet Sanitary rules and norms 2.3.2. [21] as a reference while №

581 is aligned with Commission Regulation (EC) No 2073 and is enforced since 2015. We can conclude that microbiological limits set by regulation № 581 are more recent than regulation №301/n, which follows older guidelines and specific requirements toward traditional cheese microbiological limits require revision.

Dairy industry food chain. Traditional Georgian cheese is in high demand and is an essential part of Georgian cuisine, consumed fresh or used in the traditional dishes. Dairy cattle are a central resource for peasants and contribute to household food security. Shares of family holdings in the dairy sector are at 97.5% second place after poultry, which is mainly owned by industrial farms, with 61% share[22]. The small household farms own 89% of cattle, 1 to 9 cows, while only 1% is owned the large holding with over 50 cattle heads [23]. There are 360 registered dairy business operators[24] working on raw milk collected from peasants and on importing milk powder, due to lack of milk supply or low price.

Peasants often use outdated practices and have low awareness of personnel hygiene, good hygiene practices, and veterinary control. Reasonable procedures such as hand washing, cattle hygiene before milking, equipment and utensils cleaning, milk pasteurization, storage temperature are rarely followed[25]. In addition, cattle in small farms do not receive regular veterinary control and depend on free vaccination by the Government[26]. Lack of veterinary control increases the risk of veterinary diseases and AMR development in the food chain, through incorrect usage of veterinary drugs, which can be purchased without prescription and monitoring.

A major part of cheese from all the regions ends up in Tbilisi capital of Georgia, supplying one-third of the country's population. Wholesales collecting the dairy products produced in small households deliver cheese in five main agrarian open market areas in Tbilisi: Gldani, Didube, Vagzali, Samgori, Varketili. The open market assessment during sample collection highlighted breaches of regulatory requirements and a lack of control. Which presents a direct risk for consumer health and an increased risk of pathogenic bacteria contamination[27]. Major areas for improvement are food handler hygiene and awareness, cleaning practices, good hygiene practices and cross-contamination risks, food storage temperatures, shelf-life control, food labeling. Most open markets are not provided with well-maintained handwashing facilities nearby[28][13]. Open markets are rarely equipped with water sources, necessary cleaning equipment, and chemicals, leading to insufficient cleaning practices [28][30].Dairy products are sold at ambient temperature directly from display tops or from old display units, which rarely maintain the temperature within safe storage limits [28, 31, 32]. Cross-contamination occurs between different product categories: Ready to eat, raw meat, dirty fruit, and vegetables, sold from the same display despite the separation of market areas[28]. Dairy products are sold without packaging or placed directly on dirty surfaces while the same knife, trays are used without regular cleaning[15]. Dairy products are sold without labels and shelf-life markings, indicating a lack of traceability[33][34]. In addition to open markets organized retail chains are highly concentrated in Tbilisi, with 28% of market share, expected to reach 41% by 2024[35]. Retail stores sell a major part of cheese produced by dairy factories. While big producers have a top share on market and are supplying several retails. Assessment during sample collection highlight-ed better follow-up of basic hygiene requirements, effected by frequent control from the National Food Agency [27][36]. Product storage temperature is followed[28][31][32], cross-contamination risk is reduced by keeping products in the self-service(70%) or in separated traditional displays(30%). Food labeling requirements are better followed and most products have a label with expiry dates [33][33].

Consumers awareness as a driver of the dairy industry food chain. Consumers' demand is the main driver for food business operators. The majority of the population purchase products on the open market, supporting continued trade, despite distinct unhygienic conditions. It puts an economical strain on retail stores and other food business operators who face additional expenses following the regulation. Additionally, the consumer's purchase traditions to touch and taste the product before purchase initiates practices of selling product on ambient temperature and increases cross-contamination.

Consumers' awareness is reflected in the accurate implementation of food labeling regulations as well. Regulated by the resolution №301 "Technical Regulation–on approval of the provision of food information to consumers" [19] and Resolution №152, "On the approval of the technical regulation on milk and dairy products" [33] [37]. Assessment done during samples collection highlighted that none of the open market merchants followed food labeling regulation, and only 40% had an inaccurate old label. While 100% of cheese sold in retail had labels, most

of them were missing some mandatory information. Food nutritional value indicated on labels was on average 20% higher than the ratio between proteins, carbohydrates, and fats [33]. There was no correlation between shelf life and packaging type, storage temperature, salt content, key extrinsic characteristics used for shelf-life detecting[38], which indicates lack of shelf-life study. Milk thermal treatment method was indicated only on 50% of labels, 30% of labels indicated usage of raw milk without information of the thermal treatment, while 20% of labels did not have any information. Information about milk pasteurization is vital for consumers to make conscious decisions concerning using unpasteurized dairy products with a high microbial load [37].

Low awareness of food labeling is misused in marketing, soft cheese is often sold next to its cheaper "alternatives" made from plant oil or milk powder. The violation of appellation of origin is also an example of consumers' awareness. All cheeses collected in the retail chain were named "Imereteriani" cheese when none was made in the Imereti region[39]. Following is affected by the tradition of calling soft cheese "Imeretaiani" cheese, contradicting the regulation.

Material and methods

To assess the presence of food pathogens in Georgian traditional soft cheese, we collected double cheese heads from 10 major organized retail chains[35] and 11 open markets in Tbilisi, 42 samples in total. Samples were selected randomly, avoiding collecting cheese from the same producer for diversity and when possible, prioritizing cheese from bigger cold display units on the open market.

All samples were delivered to the food microbiology laboratory and analyzed directly after purchase. Microbiological analyses methods were selected in compliance with Resolution N581[40] as follows: EN ISO 16649-2 -Horizontal method for the enumeration of beta-glucuronidase-positive *Escherichia coli* and ISO 6888-1 Horizontal method for the enumeration of CoPS. The Kirby-Bauer disc diffusion method was used to detect bacteria strains' antibiotic sensitivity[41][44]. Analyses are done in a laboratory having accreditation for all the above methods in compliance with EN ISO 17025[45].

Results of cheese microbial contamination load and antimicrobial-resistant development

Laboratory analyses showed alerting results. *Escherichia coli* was detected in 93% of samples, moreover, in 85% of samples, results were above 1000CFUs; Coagulase-positive *Staphylococci* results showed fewer colony-forming unit numbers from 300cfus up to above 300,000cfus. *CoPS* were detected in 62% of samples (Fig. 2).

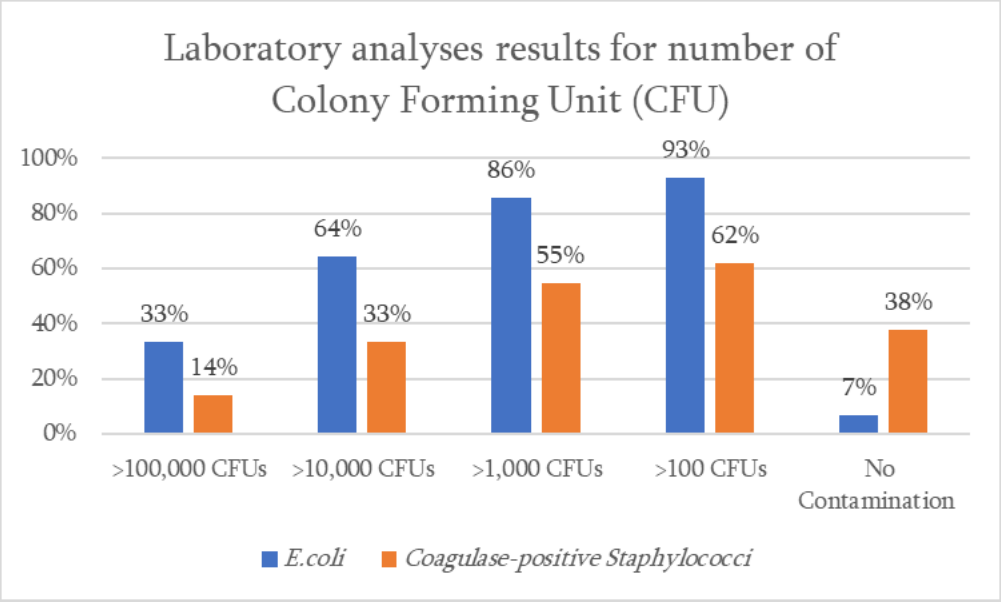

Fig. 2. *Laboratory analysis results for E. coli and CoPS number of Colony Forming Unit (CFU), N=42.*

These results can have several interpretations based on which regulations have been used for assessment. According to №301/n E. coli results directly violate limits, as coliforms are not allowed in 0.001g/cm^3 of the sample. To assess compliance of genus of Staphylococcus bacteria load with №301/n, information of production technology is required; as up to 1000cfu/g S. aureus is allowed in cheese made with the addition of bacterial culture and is prohibited in 0.1g/cm^3 of homemade cheese[46]. Nevertheless, 55% of laboratory analyses results showed higher colony-forming units of CoPS, than stated in either of the parameters.

Information concerning cheese thermal treatment is required to assess compliance of *CoPS* and *E. coli* results with №581. *CoPS* limits vary for raw milk, pasteurized, and low thermally treated cheeses. While *E. coli* limits are set only for Cheeses made from milk or whey that has undergone heat treatment [13], [40]. Without an indication of the pasteurization process on the label, we are unable to give a more detailed assessment for regulatory compliance. Nevertheless, 14% of samples violated all parameter requirements. Out of 42 cheeses, only 23% (ten samples) collected from organized retail chains indicated using pasteurized milk; 100% of the which violated requirements for *E. coli* limits, and 40% violated *CoPS* limits.

Identified *E. coli* and *CoPS* strains were tested on antimicrobial sensitivity towards eight antibiotics: Oxytetracycline; Tylosin; Trimethoprim; Enrofloxacin; Amoxicillin; Penicillin G; Streptomycin; Colistin. At least one of them contain 80% of veterinary drugs registered in Georgia for dairy cattle contain [12].

CoPS strains showed high multidrug resistance to a minimum of 3 out 6 test antibiotics. All strains show resistance to Amoxicillin, Penicillin, Enrofloxacin. At the same time, 42% of strains were resistant to Streptomycin and 35% to Trimethoprim. Oxytetracycline results were most promising, with only 19% resistance (Fig. 3).

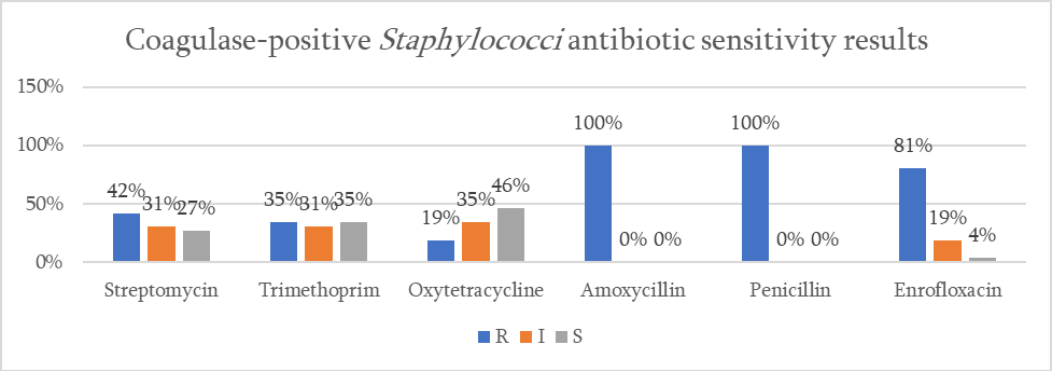

Fig. 3. Antibiotic sensitivity of *CoPS* (N=26).

E. coli strains showed multidrug resistance to three out of eight tested drugs. All strains show resistance to Amoxicillin, Penicillin, Tylosin. At the same time, 44% of strains were resistant to Streptomycin and 36% to Trimethoprim. Enrofloxacin, Oxytetracycline, and Colistin showed relatively low 8%, 15%, and 26% resistance. (Fig. 4)

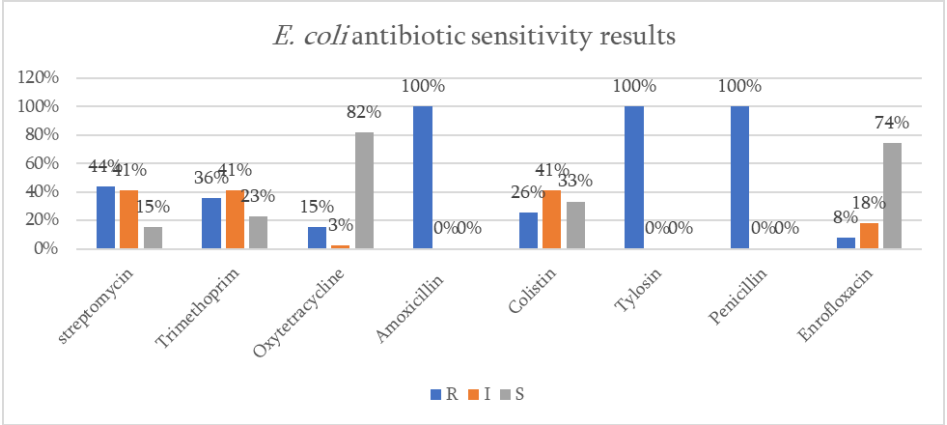

Fig. 4. Antibiotic sensitivity of *E. coli* (N=39).

Conclusion

Pathogenic bacteria contamination results and high antimicrobial resistance are alerting and indicate uncontrolled and incorrect usage of antibiotics in dairy farming and the need for hygiene and food safety requirement improvement along the full dairy food chain. In addition, microbiological limits set by the regulation and shelf-life study performance by food business operators require review. Findings are especially critical in times of rapid antimicrobial resistance, which can easily spread through the food chain to consumers. Research highlights that current agriculture, veterinary, food industry, and safety practices and regulatory controls are not sustainable for the future. The multi-sectoral approach is required to increase consumers' and farmers' awareness as key drivers of supply and market.

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