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Assessment of sodium chloride tolerance and antibiotic resistance of *Citrobacter braakii* EC-PS1 and *Macrococcus caseolyticus* Li-PT1 isolated from artisanal cheeses

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Abstract. The different varieties of cheese are fermented dairy products with high nutritional value, which have been part of the healthy human diet for centuries. Cheeses are characterized by complex and diverse microbiota carrying beneficial, spoilage, or foodborne pathogen bacteria. Harmful bacteria originate from the production chain. Identification and characterization of microorganisms in cheese are important nutritional, food safety, and technological issues. During this study, *Citrobacter braakii* EC-PS1 and *Macrococcus caseolyticus* Li-PT1 bacteria strains, isolated on selective mediums from two artisanal cheeses, were identified by way of 16S rDNA gene sequence analysis. Their antibiotic resistance and NaCl stress tolerance were also determined. *M. caseolyticus* Li-PT1 showed tolerance to 6.5% and 10% NaCl. The increasing NaCl concentration above 15% contributed to the decrease of viability in bacteria. The increasing NaCl concentration to 10% contributed to the decrease of viability in *C. braakii* EC-PS1 bacteria. The identified bacterial species exhibited different levels of resistance to the tested antibiotics. *C. braakii* EC-PS1 showed resistance to chloramphenicol, ampicillin, and streptomycin, whereas *M. caseolyticus* Li-PT1 was susceptible only to two antibiotics (erythromycin, tetracycline) out of the eight tested.

Keywords and phrases: osmotic stress, antibiotic resistance, cheese, food safety

1. Introduction

Tolerance to food-processing-, production-, or storage-associated stress conditions contributes to the survival of bacteria in adverse environmental circumstances. Foodborne pathogens (*Salmonella* spp., *Escherichia coli*, *Campylobacter jejuni*, *Listeria monocytogenes*) are able to adapt effectively to stressful conditions (*Bucur et al.*, 2018; *Oh et al.*, 2019; *Kim et al.*, 2021; *Guillén et al.*, 2020; *Guillén et al.*, 2021; *Duze et al.*, 2021).

The bacterial cells sense the stress on molecular level and respond to these conditions with specific stress responses, which enable bacteria to respond to particular suboptimal conditions in their immediate surroundings or with general stress responses (*Begley & Hill*, *2015*).

Some stress factors, including heat treatment, cooling/freezing, acidic conditions, or osmotic tolerance, may induce in several bacteria (*L. monocytogenes*, *E. coli*, and *Salmonella* spp.) the so-called cross-protection phenomenon (*Begley & Hill*, 2015; *Chen*, 2017).

Osmotic stress causes physiological or morphological changes in the cell of foodborne pathogens, inhibiting their growth or leading to cell death. The mitigation of the adverse effects of osmotic stress includes the modification of membrane proteins and transportation systems. The resistance to high osmotic habitats is rare in bacteria. These stress conditions contribute to the alteration of the osmoregulation of the cell resulting in the deterioration of metabolic activities (*Ding et al.*, 2022).

Safe food production implies that foodborne pathogens are exposed to high osmolarity conditions. Many bacteria species in these environments respond with persistence and adaptation regarding the morphology, growth dynamics, or biofilm development (*Sleator & Hill*, 2001; *Ding et al.*, 2022).

The reply of the cells consists in the accumulation and release of osmolytes as organic molecules (trehalose, proline, trimethylammonium compound, glycine betaine, and carnitine) or inorganic ions (K^+) through active transport. In case of high osmolality, the compatible compounds prevent dehydration of the bacterial cell. The accumulated osmoprotectants alter minimally the physiology and cellular functions. Different factors are involved in response as transporters, multiple enzymes and channels with redundant specificities and functions (*Sleator & Hill*, 2001; *Wood*, 2015; *Bremer & Krämer*, 2019; *Ding et al.*, 2022).

Reaction to osmotic challenges in Gram-positive and Gram-negative bacteria is via potassium ion uptake stimulation, which contributes to stabilizing the net negative charge of the macromolecules in the cytoplasm (*Sleator & Hill*, 2001). The mitigation of hyperosmotic stress was associated with lysine-to-α-aminoadipic-δ-semialdehyde pathways in *Silicibacter pomeroyi* (*Neshick et al.*, 2013). In *Streptomyces coelicolor,* several transcriptional regulations of enzymes are involved in the alleviation of stress conditions (*He et al.*, 2018).

Growth in high-salt media implies the modification of cytoplasmic membrane fatty acid compositions, related to the phospholipids' head group, too. It has been shown that *E. coli* respond to osmotic stress with an electrolyte due to growth in the proportion of cardiolipin and to the reduction of phosphatidylglycerol content. In non-electrolyte-induced osmotic stress, the phosphatidylglycerol content was not altered (*Romantsov et al.*, 2009). In *Enterococcus* spp. strains, the changing sodium chloride (NaCl) concentration (4%, 7%) altered the expression level of genes associated with virulence factors (*Zarzecka et al.*, 2022).

The different types of cheese belong to the group of fermented dairy products, which have been part of healthy human diet for centuries, containing essential nutrients and bioactive substances. Cheese consumption in many countries has increased significantly in recent years. In the European Union, about 36% of milk is used for cheese production (*de Oliveira et al.*, 2017). Cheeses are characterized by complex and diverse microbiota carrying beneficial, spoilage, or foodborne pathogen bacteria. The harmful bacteria originate from the production chain. Cheese is considered a valued product providing beneficial or harmful health effects. The negative effect is associated with the presence of foodborne pathogens. Raw milk cheese can be a source of human pathogenic bacteria such as *Salmonella* spp., *Streptococcus* spp., *L. monocytogenes*, *Helicobacter pylori*, *Campylobacter* spp., *Escherichia coli*, *Coxiella burnetti*, *Mycobacterium* spp., *Brucella* spp., *Staphylococcus aureus*, *Arcanobacter pyogenes*, *Bacillus cereus*, *Leptospira*, *Clostridium* spp., and *Yersinia enterocoliticia*. Unsuitable cheese-making conditions favour the growth and development of these species (*O'Sullivan & Cotter*, 2017).

The fermented foods without heat treatment could be a source of antibiotic-resistant bacteria. The uncontrolled use of antibiotics in dairy cattle contributed to the spread of antibiotic-resistant bacteria. *Staphylococcus* strains found in Minas Frescal cheese were shown to exhibit resistance to penicillin, oxacillin, and clindamycin and harbour antibiotic resistance genes such as blaZ, mecA, lsaB, msrA, and ant4 (*da Silva Abreu et al.*, 2020).

Different bacteria species belonging to the *Enterococcus* genus, such as *E. faecium*, *E. faecalis*, and *E. durans*, were detected in Protected Designation of Origin (PDO) cheese from six cheese-making units. Several bacteria strains showed antibiotic resistance to β-lactams, aminoglycosides, glycopeptides, quinupristin-dalfopristin, teicoplanin, and tetracycline (*Rocha et al.*, 2022).

Infections were caused by *Salmonella* species, including multi-resistant serotypes originated from cheeses (*Cogan*, 2011; *de Oliveira et al.*, 2017). Raw milk or raw milk cheese contaminated by enterotoxigenic *Staphylococcus* spp. can be considered in many cases the source of staphylococcal enterotoxin. In Brazilian cheese samples, they identified bacteria showing resistance to clinically relevant antimicrobials such as penicillin, cefoxitin, oxacillin, clindamycin, erythromycin, tetracycline, tobramycin, gentamicin, ciprofloxacin, chloramphenicol, and also community-acquired methicillin-resistant *Staphylococcus aureus* (*Aguiar et al.*, 2022). Methicillin resistance was found in coagulase-negative staphylococci isolates and in *Macrococcus caseolyticus* (*Klempt et al.*, 2022). *M. caseolyticus* with casein-hydrolysing capacity was found in the bacterial community of cow's milk in artisanal cheeses from north-western Argentina (*Suárez et al.*, 2020).

Several studies reported that cheese produced from raw and heat-treated milk was associated with foodborne outbreaks caused by infection of *L. monocytogenes*, *S. aureus*, *Salmonella* spp., Shiga-toxin producing *E. coli*, *Campylobacter* spp., *Brucella* spp., *Shigella* spp., *Clostridium perfringens*, and *Bacillus cereus* (*van Asselt et al.*, 2017; *Adhikari et al.*, 2018).

Bacterial growth and development in various cheese types are controlled by different environmental factors (water activity, pH, ripening temperature, redox potential), composition (NaCl content, nitrates), metabolites, and bacteriocins. There are few studies on the prevalence, stress tolerance, and antibiotic resistance of spoilage and foodborne bacteria in locally available dairy products. The aim of the present study was the determination of the antibiotic resistance and NaCl stress tolerance of *Citrobacter braakii* EC-PS1 and *Macrococcus caseolyticus* Li-PT1 originated from cheese.

2. Materials and methods

During the study, the microbial contamination of salty type soft cheese and fresh cheese originated from open air markets, with cultivation methods on different selective media (ChromoBio TBX, Listeria Mono Differential Agar Base), was determined. Bacterial colonies with the highest number and with a characteristic colony morphology were isolated, and pure cultures were obtained. Identification of isolated bacteria strains was done using 16S rDNA gene sequence analysis (*György et al.*, 2021).

The antibiotic susceptibility testing of the isolated bacteria was performed with the disk diffusion method, according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. A total of eight different antibiotic disks containing the antibiotics erythromycin 15 µg, chloramphenicol 30 µg, kanamycin 30 µg, ampicillin 10 µg, clyndamicin 2 µg, streptomycin 10 µg, tetracycline 30 µg, and gentamicin 10 µg were used (*György et al.*, 2021).

The tested bacterial species were grown for 28 hrs at 37°C and inoculated in nutrient broth with 2%, 4%, 6,5%, 10%, 15%, 20%, and 25% NaCl content. The optical density values were recorded at the wavelength of $\lambda = 595$ nm by Fluostar Optima Microplate Reader (BMG Labtech, Ortenberg, Germany) in every 4 hrs. Growth curves were obtained from the absorbance values. Bacterial survival rate was calculated according to *Nath et al.* (2020) with slight modifications.

3. Results and discussion

The emergence of antibiotic-resistant and NaCl stress-resistant foodborne pathogens has created great pressure on the food industry during processing and storage. The two identified bacterial species were isolated on the selective mediums ChromoBio TBX and Listeria Mono Differential Agar Base from salty type soft cheese and also from fresh cheese samples.

According to the results of the 16S rDNA sequence analysis, one of the bacterial isolates belongs to the *Macrococcus* genus, *M. caseolyticus*, previously classified as *Staphylococcus caseolyticus* and showing 99.27% similarity, whereas the other bacteria belong to *Citrobacter genus, C. braakii* EC-PS1 with 99.6% similarity.

The *Macrococcus* genus taxonomically belongs to the family of *Staphylococcaceae* and comprises eleven species as follows: *Macrococcus bovicus*, *M. carouselicus*, *M. equipercicus*, *M. brunensis*, *M. hajekii*, *M. lamae*, *M. goetzii*, *M. epidermidis*, *M. bohemicus*, *M. caseolyticus*, and *M. canis.* Several studies reported isolation and identification from dairy (goat coalho cheese, Amazonian artisanal cheeses, raw milk) and meat products (fermented liver sausage). Alkaline-fermented foods with high protein content were also a source of *M. caseolyticus.* This is not considered a human pathogen but a relevant harmful bacteria causing infections in veterinary medicine (*Martins et al.*, *2018*; *Mazhar et al.*, 2018; *Ouoba et al.*, 2019; *Ribeiro-Júnior et al.*, 2020; *Arãgao et al.*, 2022; *Belleggia et al.*, 2022).

The Gram-positive bacteria were commonly isolated from commercially available animal meat and characterized with susceptibility to photocatalytic disinfection by nano-TiO_2 (*Wang et al.*, 2014). The insect products as meat alternatives originated from Belgian markets contained *M. caseolyticus* besides *E. faecium* (*Geeraerts et al.*, 2020). The nitrite content of milk powder was associated with the presence of thermophilic bacteria. Milk from a dairy processing plant was a source of *M. caseolyticus* with nitrate-reducing ability (*Wong & Flint*, 2019). *M. caseolyticus* was also a dominant species of bacterial flora of smoked fish (*Maïworé et al.*, 2021).

The facultative anaerobic Gram-negative *Citrobacter* spp. belongs to the family *Enterobacteriaceae* (*Liu et al.*, 2021). *Citrobacter braakii* was detected also by others in cheese samples. The predominant species of ewe milk and curd contained *C. braakii*, *C. freundii*, and *Klebsiella oxytoca.* The gas production of *C. braakii* was associated with early blowing in soft and semi-hard ewe cheeses (*Tabla et al.*, 2016). Examination of microbiota of the sheep cheese "bryndza" detected the presence of *C. braakii* (*Kačániová et al.*, 2019).

M. caseolyticus Li-PT1 growth was stimulated with 2% and 4% NaCl content, and the bacterial survival rate exceeded 100%. *M. caseolyticus* Li-PT1 showed tolerance to 6.5% and 10% NaCl (*Fig. 1*), with 96.07% and 64.71% survival rate. Increasing the sodium chloride concentration above 15% contributed to the decrease of viability in bacteria – the rate of survival was 49.56%. The identified bacteria strain also showed reduced growth values at 20% and 25% NaCl concentration, with survival rates of 40.83% and 47.92%.

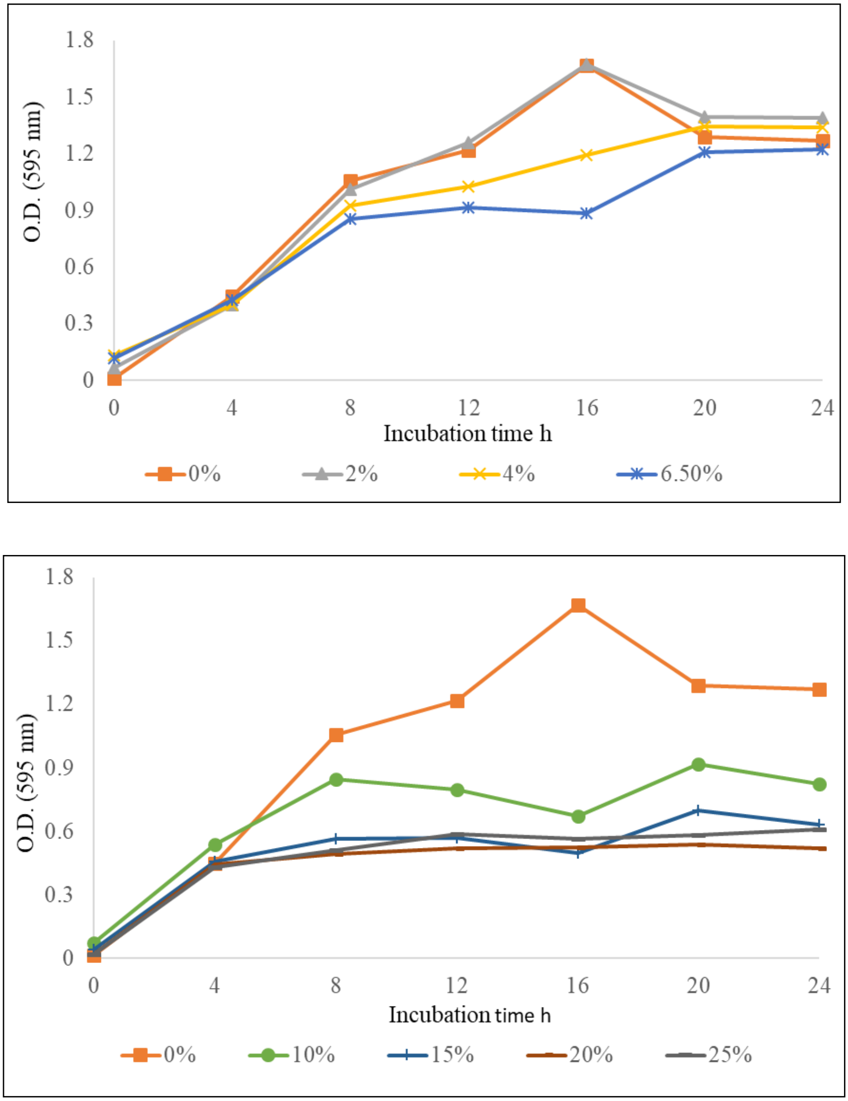

Figure 1a–b. Growth curves of *Macrococcus caseolyticus* Li-PT1, grown in media containing different concentrations of NaCl

The growth of *C. braakii* EC-PS1 was repressed by increasing the NaCl concentration of the growth medium (*Fig. 2*). As mentioned in the case of *M. caseolyticus* Li-PT1, *C. braakii EC-PS1* showed tolerance to 4% NaCl.

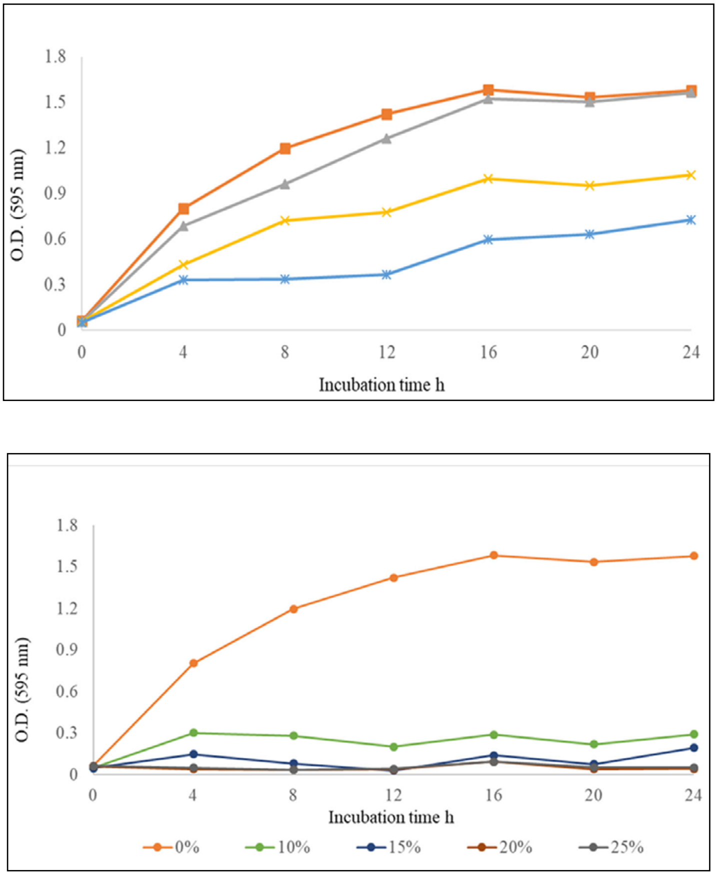

Figure 2a–b. Growth curves of *Citrobacter braakii* EC-PS1, grown in media containing different concentrations of NaCl

Increasing the NaCl concentration to 10% contributed to the decrease of viability in *C. braakii* EC-PS1 bacteria. These are also confirmed by the bacterial survival rate, which is equal to 99.02% in the presence of 2% NaCl, 64.68% in the presence of 4% NaCl, and 45.92% in the presence of 6.5% NaCl. The identified bacteria strain showed a minimal sign of growth at 10% NaCl, with a bacterial survival rate of 18.47%, and no sign of growth after 4 hrs of incubation above 10% NaCl.

Table 1. The antibiotic resistance pattern of the two identified bacterial species originating from dairy products

	Erythromycin 15	**Chloranphenicol 30**	**Kanamycin 30**	**Ampicillin 10**	**Clindamycin 2**	**Streptomycin 10**	**Tetracycline 30**	**Gentamicin 10**
Citrobacter braakii EC-PS1	N	R	S	R	N	R	S	I
Macrococcus caseolyticus Li-PT1	S	R	R	R	R	R	S	R

The identified bacterial species exhibited different levels of resistance to the tested antibiotics. *C. braakii* EC-PS1 showed resistance to chloramphenicol, ampicillin, and streptomycin (*Table 1*). *M. caseolyticus* Li–PT1 was susceptible only to two antibiotics (erythromycin, tetracycline) out of the eight tested.

The safety evaluation of microorganisms in the food chain represents a priority. One of the main public health concerns is the possibility of the resistance plasmids' transfer through the food chain to pathogenic and/or commensal bacteria, as in the case of *M. caseolyticus.* A whole-genome analysis showed multidrug resistance and methicillin resistance determinants in *M. caseolyticus* (*Mazhar et al.*, 2018). *M. caseolyticus* harbours different genes conferring resistance, such as mecA, mecB, mecC, and mecD. It has been demonstrated that bacteria exhibit gene-sharing ability between species.

Our findings regarding origin and antibiotic resistance are in concordance with other studies. *M. caseolyticus* from cheese samples showed resistance to cefoxitin, penicillin G, harbouring also methicillin resistance genes. Results suggest that this is a food safety concern because of the broad-host dissemination of the mecD gene (*Klempt et al.*, 2022). In milk samples originating from cows with subclinical mastitis, it was detected *M. caseolyticus* bacteria showing antibiotic resistance towards ampicillin, cefoxitin, erythromycin, oxacillin, and penicillin (*de Oliveira et al.*, 2022).

It was found that Gram-negative *C. braakii* rarely causes infections in relation to *C. freundii* and *C. koser.* Despite this, *C. braakii* was associated with bacteremia in a cervical cancer patient and with infections in immunocompromised patients (*Hirai et al.*, 2016; *Oyeka & Antony*, 2017), and it was isolated also from a patient treated with intravenous antimicrobial therapy (*Fernández-Polo et al.*, 2021). Regarding antibiotic resistance, *C. braakii* isolated from patients with extraintestinal infections was characterized as multidrug-resistant (*Liu et al.*, 2021).

4. Conclusions

The presence of antibiotic-resistant bacteria with stress tolerance in food production is a food safety concern. The tested dairy products contain antibiotic-resistant bacteria. The results obtained indicate that one bacterial strain could grow in sodium chloride stress conditions. Further investigation is necessary to reveal molecular responses of the bacterial strains to stress factors to prevent or control their growth.

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