Phenotypic, Biochemical and Genotypic Charcaterisation of Some Factors Involved in the Virulence and Survival of Bacteria Isolated from Food and Food Processing Surfaces

MIHAELA MAGDALENA MITACHE[1,2]*, CARMEN CURUTIU[3], IRINA GHEORGHE[3], VERONICA LAZAR[3,4], MARCELA BUCUR[3,4], CORALIA BLEOTU[5], DANIEL COCHIOR[1], ELENA RUSU[1], CARMEN MARIANA CHIFIRIUC[3,4]

[1]University Titu Maiorescu of Bucharest, Faculty of Medicine, 67A Gheorghe Petrascu Str., Bucharest, Romania

[2]Public Health Direction Bucharest, 72-74 Avrig Str., Bucharest, Romania

[3]University of Bucharest, Faculty of Biology, Department of Microbiology, 91-95 Splaiul Independentei, 050095, Bucharest, Romania

[4] Research Institute of the University of Bucharest, 91-95 Splaiul Independentei, 050095, Bucharest, Romania

[5] Stefan S. Nicolau Institute of Virology, 285 Mihai Bravu Road, 030304, Bucharest, Romania

Species of the Enterobacteriaceae family are frequently involved in various gastrointestinal infectious diseases, including food poisoning that without proper treatment and medical supervision can be fatal for some patients, particularly for those with weak immune systems. Therefore we proposed to characterize the virulence factors of some strains from the Enterobacteriaceae family isolated from food and food processing surfaces at the phenotypic and genotypic level in order to assess the microbiological risk for the public health. The strains were identified using conventional biochemical methods. The expression of virulence soluble markers and the ability of the enterobacterial strains to adhere to the inert and cellular substrate were investigated. At the genetic level the presence of some genes involved in adhesion and virulence was also investigated by PCR. The tested strains revealed a different adherence capacity to the inert and cellular substrata and also ability to develop biofilms. Regarding enzymatic factors, esculin hydrolysis with the production of esculetin as an iron chelating agent and the casein hydrolysing protease were mostly expressed. The results proved that Enterobacteriaceae strains isolated from food could represent a microbiological risk factor for consumers' health, contributing also to the setting up of the reservoir virulence and resistance genes.

Keywords: food control, Enterobacteriaceae, virulence genes reservoir, surveillance, public health

Foodborne and waterborne diarrhoeal diseases kill an estimated 2.2 million people annually, most of them being children. The most common symptom of foodborne illness is the acute diarrhoea, but sometimes some other serious consequences, such as kidney and liver failure, brain and neural disorders, reactive arthritis, cancer and death could appear.

Enterobacteriaceae group has an epidemiological interest and importance as some of them are pathogenic and may cause serious infections and/or food poisoning [1]. The *Enterobacteriaceae* family is represented by a widely distributed, large group of Gram-negative non-spore-forming, aerobe, facultative anaerobe bacteria. Some of these species are harmless commensals, such as some strains of *E. coli*, but other species from this family are important human and animal pathogens (enterotoxigenic, enteropathogenic, enteroaggregative, and entero-haemorrhagic *Echerichia coli* pathotypes), and some are pathogenic to plants and insects.

Due to their broad spread, inevitably some members of the *Enterobacteriaceae* will enter in the food chain. A very good example is offered by *E. coli* O157:H7, which has become one of the most important foodborne pathogens. Members of the family are also responsible for food spoilage and therefore contribute to substantial economical losses and food wastage. Due to their involvement in the food chain, members of *Enterobacteriaceae* family provide a valuable role as indicator organisms in processed foods representing a measure of food quality and spoilage potential.

The initial *Enterobacteriaceae* contamination level in the raw materials is predominantly governed by Good Agricultural Practices (GAP) during primary production and subsequently during slaughter of livestock at the abattoir. Further along the food supply chain, contamination by *Enterobacteriaceae*, including pathogens, must be prevented or controlled by the application of one or more of the acknowledged quality assurance systems including Hazard Analysis and Critical Control Point (HACCP) systems and Good Manufacturing Practices (GMP) [2].

A current concern is the fact that some *Enterobacteriaceae* members have emerged or could potentially become pathogenic as a result of the acquisition of virulence associated genes (toxins, colonization factors) carried on mobile genetic elements such as transposons, plasmids, insertion sequences and bacteriophages, transfer that can be achieved without genus and species barrier [3].

The aim of the study was to characterize the resistance and virulence factors of some *Enterobacteriaceae* strains isolated from food and food processing surfaces at the phenotypic and genotypic level in order to assess the microbiological risk for the public health.

Experimental part

Materials and methods

The strains were isolated according to ISO standards. The strains were isolated according to SR ISO 18593:2007 (the horizontal sampling method using swabs taken from

* email: magda.mitache@yahoo.com

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surfaces in the food industry environment- food processing units), SR ISO 4831:2009 (for detection and enumeration of coliform bacteria in products intended for human consumption or animal feed, by calculating the most probable number), SR ISO 4832:2009 and SR ISO 21528-2:2007 (horizontal method for detection and enumeration of *Enterobacteriaceae* strains), SR ISO 16649-2:2007 (horizontal method for the enumeration of *E. coli* beta-glucuronidase positive- colonies grown at 44^0C using 5-bromo-4-chloro-3-indolyl beta-D-glucuronide). The identification was realized using biochemical conventional tests [4].

Evaluation of the biofilm development on inert substrata was assessed by the microtiter method. Over night bacterial cultures were grown in 96 multi-well plates containing Tryptic Soy Broth (TSB) for 24h, 48h and 72h at 37°C. After each period of time, the plates were subsequently emptied and washed three times with phosphate buffered saline (PBS). The adherent cells were then fixed with cold methanol, stained with an alkaline 1% violet crystal solution for 15 minutes, washed with water and resuspended in a 33% acetic acid solution. The intensity of the suspension was spectrophotometrically assessed, the amount of adhered biomass being proportional to the absorbance value read at 492 nm.

Cell adherence assay was perform using Cravioto's adapted method. The HeLa cells monolayers were washed three times with PBS and 1 ml of fresh medium without antibiotics was added to each well. The suspension of enterobacterial strains from bacterial mid-logarithmic phase cultures grown in nutrient broth was adjusted to 10^8 cells/ml and 1 ml was used for the inoculation of each well. The inoculated plates were incubated for 2 h at 37°C. After incubation, the monolayers were washed three times with PBS, briefly fixed in cold methanol (3 min), stained with Giemsa solution (1:10) for 20 min. The plates were examined microscopically to evaluate the adherence index and patterns. The adherence index was expressed as the ratio between the number of the eukaryotic cells with adhered bacteria and 100 eukaryotic cells counted on the microscopic field. The adherence patterns were defined as: localized adherence (LA) when tight clusters of microorganisms were noticed on the HeLa cell surface, aggregative adherence (AA) when a microbial stacked brick pattern characterize the attachment, diffuse adherence (DA) when the bacteria adhered diffusely, covering the whole surface of the cell [5].

The bacterial virulence phenotype was assessed by performing enzymatic tests for the expression of some virulence soluble factors. Over night culture of the strains were evaluated for the following virulence factors expression: haemolysins, other pore forming toxins (lecithinase, lipase), proteases (caseinase), and aesculin hydrolysis [6]. Detection of haemolysin production was performed by spotting the fresh cultures on 5% sheep blood agar medium and incubation at 37°*C* for 24h. The colorless area around the culture revealed the presence of haemolysis activity. For lipase production the strains were spotted on 1 % Tween 80 agar as a substrate and followed by incubation at 37°C for 24h. An opaque (precipitation) zone around the spot was registered as positive reaction; for lecithinase production, the cultures were spotted into 2.5% yolk agar and incubated at 37°C for 24h. A clear zone around the spot indicated the lecithinase production. The caseinase activity was determined using 15% soluble casein agar as substrate. The strains were spotted and after incubation at 37°C for 24h, a white precipitate surrounding the growth indicating casein proteolysis. For the aesculin hydrolysis the medium containing Fe^{3+} citrate was used and inoculated by spotting and incubated for 24h at 37°C. A black precipitate arround culture due to esculetol released under the action of beta-galactosidase was considered positive reaction.

The antibiotic susceptibility testing was performed by Kirby-Bauer standard disk diffusion method (CLSI, 2014). The following antibiotics were tested: gentamycin (CN) (10μg), sulfamethoxazole-trimethoprim (STX) (1.25/23.75μg), ampicillin (AMP) (10μg), amoxicillin-clavulanic acid (AMC) (20/10μg), tetracycline (TE) (30μg), cefotaxime (CTX) (30μg), ceftazidime (CAZ) (30 vg), imipenem (IMP) (10 μg), amikacin (AK) (30 μg), tobramicin (TOB) (10 μg), ciprofloxacin (CIP) (5 μg), cefamandole (MA) (30μg). The results were recorded after 24 h incubation at 37°C.

The genetic support of the virulence factors mediated by enzymatic mechanisms was investigated by PCR for all *Enterobacteriaceae* strains using a reaction mix of 20 or 25 μL (PCR Master Mix 2x, Thermo Scientific) containing 1 μL of DNA obtained by the thermal lysis of bacterial cell suspension. *Enterobacteriaceae* strains were investigated for the presence of genes pldA and HelD with virulence role, but also specific genes for the main pathotypes of *E. coli*: eaea, bfpA and eaf specific genes for entero-pathogenic *E. coli* strains; AggR, EAggE and EAST1 specific genes for enteroaggregative *E. coli* strains; VT1 and VT2 specific genes for enterohaemorrhagic *E. coli* strains. Also, we performed a multiplex PCR for the detection of the

Table 1

PRIMER SEQUENCE USED FOR VIRULENCE FACTORS SCREENING IN ORDER OF EXPECTED AMPLICONS SIZE

Gene	Nucleotide sequence (5'to 3')	Amplification conditions	Size (bp)	Source
eaea	EaeA-F: GGCTCAATTTGCTGAGACCACGGTT EaeA-R: GCAAATTTAGGTGCGGGTCAGCGTT	94ºC-1m; 65ºC-1m; 72ºC-1m (35x)	494bp	Designed by Chifiriuc, 2004
BfpA	BfpA-F: CAATGGTGCTTGCGCTTGCT BfpA-R: GCCGCTTTATCCAACCTGGT	94ºC-1m; 65ºC-1m; 72ºC-1m (35x)	324bp	Designed by Chifiriuc, 2004
eaf	Eaf-F: CAGGGTAAAAGAAAGATGATAA Eaf-R: TATGGGGACCATGTAATTATCA	94ºC-1m; 52ºC-1m; 72ºC-1m (35x)	397bp	Designed by Chifiriuc, 2004

AggR	AggR-F: CGATGTATACACAAAAGAAGGA AggR-R: GCCTAATGAAATATGATGGTACT	94ºC-1m; 56ºC-1m; 72ºC-1m (35x)	640bp	Designed by Chifiriuc, 2004
EaggE	EAggE-F: CTGGCGAAAGACTGTATCAT EAggE-R: CAATGTATAGAAATCCGCTGTT	94ºC-1m; 56ºC-1m; 72ºC-1m (35x)	630bp	Designed by Chifiriuc, 2004
afa, pap, sfa (multiplex PCR)	Pap-F: GACGGCTGTACTGCAGGGTGTGGCG Pap-R: ATATCCTTTCTGCAGGGATGCAATA Afa-F: GCTGGGCAGCAAACTGATAACTCTC Afa-R: CATCAAGCTGTTTGTTCGTCCGCCG Sfa-F: CTCCGGAGAACTGGGTGCATCTTAC Sfa-R: CGGAGGAGTAATTACAAACCTGGCA	94ºC-2m; 65ºC-1m; 72ºC-2m (25x)	pap-328bp afa-750bp sfa-410bp	Bouguenec & al., 1992
VT1	VT1-F: GAAGAGTCCGTGGGATTACG VT1-R: AGCGATGCAGCAGCTATTAATAA	94ºC-1m; 56ºC-1m; 72ºC-1m (35x)	130bp	Designed by Chifiriuc, 2004
VT2	VT2-F: AAGAAGATGTTTATGGCGGT VT2-R: CACGAATCAGGTTATGCCTC	94ºC-1m; 56ºC-1m; 72ºC-1m (35x)	346bp	Designed by Chifiriuc, 2004
EAST1	EAST1-F: CCATCAACACAGTATATCCGA EAST1-R: GGTCGCGAGTGACGGCTTTGT	94ºC-1m; 56ºC-1m; 72ºC-1m (35x)	111bp	Designed by Chifiriuc, 2004
pldA, helD (multiplex PCR)	pldA-F: CAG GGC TGG TTG TTG CCG GT pldA-R: AC GCC ACA GCG GAA ATG helD-F: GGT TGC TGG CGC GTG GTG AA helD-R: GCG TGA GGC AAG ACG ACG CT	95º-1m; 60º-40s; 72º-1m (30x)	pldA-284bp helD-370bp	Designed by Panus, 2012

operons pap, afa and sfa encoding adhesion factors (table 1).

Results and discussions

The adherence capacity to the inert substrate is a major problem for the food industry, because bacteria can adhere and form biofilms on the working surfaces in the food industry, and thus the foods processed on such areas may be contaminated. Biofilms are microbial communities attached to surfaces and encased in an extracellular matrix secreted by them. Biofilms could develop everywhere and virtually on every natural and man-made surface [7]. The physical structure of biofilms varies greatly from smooth, thin layers to structures like columns and mushrooms. In some cases, biofilms are populated by a single species, while in others biofilms covers a diverse microbial species. For residents of a biofilm, a community development offers significant advantages. For example, bacteria living in biofilms are significantly more tolerant to antibiotics and antibacterial substances and receive shelter from environmental stress factors, including host immune system attack. Furthermore, increased cell proximity facilitates horizontal gene transfer and sharing metabolic products between community members [8]. Biofilms present a particular interest in the context of food hygiene, therefore many studies for understanding the mechanisms underlying their formation, but also to prevent them were performed [9]. The type of materials used for food contact surfaces (e.g., stainless steel, glass, rubber, polyurethane, teflon, nitrile butyl rubber and wood) could influence the microbial adherence capacity. Besides the material itself other design aspects, such as welding, joints, corners, and equipment design could also be important factors influencing biofilm formation. In addition to these extrinsic factors, specific response of each bacterial strain with respect to a substrate is probably due to some intrinsic factors related to the cell wall, such as adhesins, proteins of the cell wall, extracellular polymers [10].

Taking into account the economic aspects of the problem, and considering the magnitude of the concerns in this area, the study of the adhesion capacity of the bacterial strains isolated from food processing surfaces,

although not a required indicator by the standards applied today, is clearly a research necessity.

All the *Enterobacteriaceae* strains isolated from food and food chain surfaces were able to adhere to the inert substrata, and also to develop biofilm. A percentage of 66% of the strains showed an increasing dynamic on the observation period, with an early stage of biofilm development at 24 h and a maximum at 72 h. The rest of the strains showed a decrease of biofilm cells at 48 h (O.D. values lower that those obtained at 24h), followed by an increase at 72 h (fig. 1).

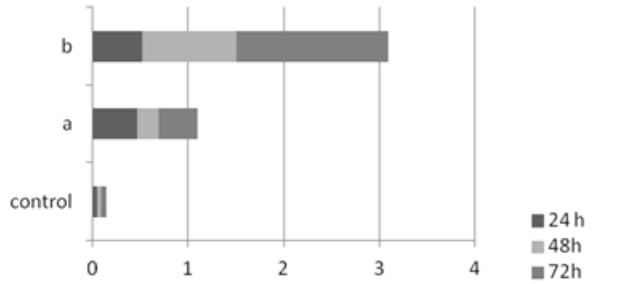

Fig. 1. Graphic representation of biofilm development at 24, 48 and 72 h

The capacity of attachment to a surface was also observed on cellular substrata, all the tested strains being able to adhere to HeLa cells, the most predominant pattern being a diffuse adherence, or localized–diffused adherence. Regarding adherence index, it proved to be ranging between 10 and 100% (fig. 2). The *E. coli* strains showing a diffuse pattern (DAEC- Diffusely adherent *E. coli*), form a heterogeneous group of microorganisms with varying virulence, which are divided into two classes, i.e. those which have fimbrial adhesins (AFA) and those possessing an adhesin involved in diffuse adherence and which is a potential cause of infantile diarrhoea [11]. Moreover, it

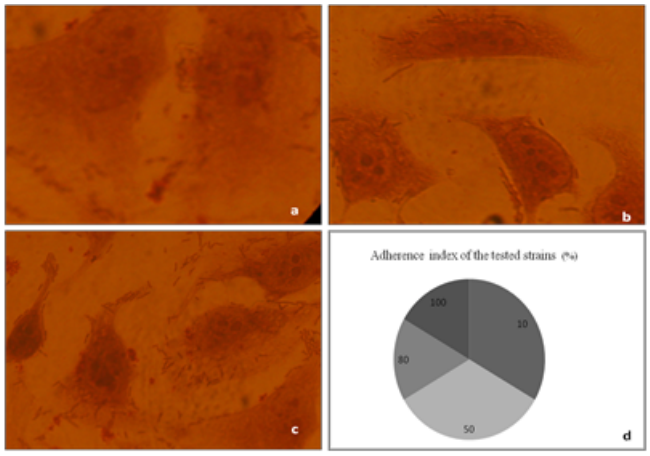

Fig. 2. Adherence to HeLa cells: (a) Control HeLa cells; (b) Diffuse adherence pattern;(c) Localized-diffuse adherence; (d) Adherence index of the tested strains to HeLa cells

seems that DEAC strains, similar to enteroaggregative *E. coli* ones (EAEC), induce the synthesis of IL-8 by epithelial cells, a chemokine with an important role in causing the diarrheal syndrome, particularly in children, due to the chemo-attractant effect on neutrophils [12]. The DAEC strains could contain one or more homologues of the locus of the enterocyte effacement characteristic to EPEC (proteins homologous to the EspA, EspB, and EspD proteins), which are necessary for signal transduction events inducing attaching and effacing lesions (A/E) leading to the formation of pedestals and/or extended surface structures phenotypically similar with those formed by the enteropathogenic and some enterohemorrhagic *E. coli* strains carrying the LEE pathogenicity island. These aspects may contribute to the pathogenic potential of DAEC strains [13].

The analysis of the soluble virulence factors showed that the enterobacterial strains express some enzymatic factors, like aesculin hydrolysis and the production of esculetin as iron chelating agent (83% from the strains), followed by caseinase (33%) and lecithinase, lipase, starch hydrolosis and beta haemolysins (16%) (fig. 3).

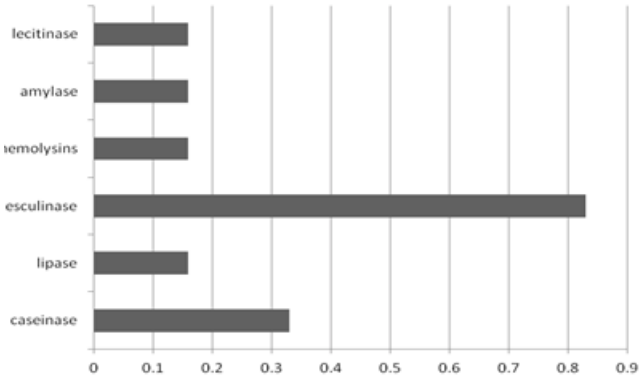

Fig. 3. Graphic representation of the level of enzymatic virulence factors

The tested strains mostly produced esculinase. Iron is an essential compound for mictobial growing and virulence. In the extracellular medium iron is found in a non assimilable form, the bacteria requiring siderophores to acquire it. It was proved that iron may be fixed by esculetol with high affinity; therefore esculinase has an important role in ensuring Fe uptake required for the activation of bacterial genes and expression of some virulence factors [14].

Caseinase is a proteolytic enzyme that hydrolyses casein, a protein abundant in milk. Numerous studies have shown that proteases produced by pathogenic organisms may contribute to the severe symptoms of infection [15].

Amylase is an enzyme produced by numerous microorganisms, capable of hydrolyzing starch. This enzyme is involved in breaking the complex sugars to monosaccharides, which are necessary for colonization of any type of tissue, but also for their survival.

Lecithinase, lipase, and beta- haemolysins are pore forming toxins that cause pores in the cell membrane, allowing the dissemination of infection [16].

The presence of all these enzymes is proving the pathogenic potential of these strains after the ingestion of the contaminated food, particularly by a immunodepressed person.

All the *Enterobacteriaceae* strains screened for the genotypic markers characteristic for the main *E. coli* intestinal pathovars and also for the pldA and HelD genes proved to negative. The pldA protein could be regarded as an important virulence factor, because it confers *E. coli* strains the ability to lyse different host cell types [17] , while the helicase IV, encoded by the helD gene product was shown to facilitate bacterial dissemination, through the degradation of extracellular DNA accumulated in different viscous secretions.

All enterobacterial strains were susceptible to almost all antibiotic tested gentamycin, sulfamethoxazole-trimethoprim, ampicillin, tetracycline, ceftazidime, imipenem, amikacin, tobramycin, ciprofloxacin and cefamandole. However, 33% of the strains showed resistance to amoxicillin-clavulanic acid and cefotaxime (66 %) (fig. 4).

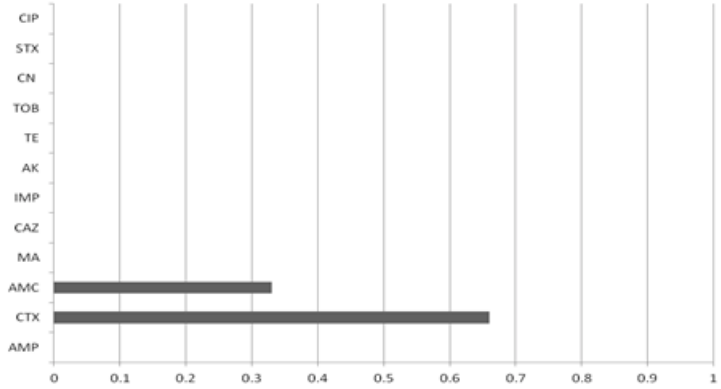

Fig. 4. The results of the antibiotic susceptibility testing

Conclusions

Our results have revealed that the enterobacterial strains isolated from food products and food processing surfaces analyzed in this study showed the potential to initiate an infection in the human or animal host, as well as the ability to persist in the food chain, facilitated by their good capacity to adhere to the inert and cellular substratum. This could either increase the risk of emerging a food disease, or contribute by food spoilage to substantial economical losses. Although these strains showed no significant resistance profiles, the need of the improvement of the effective monitoring of all stages of the food chain should remain a research focus, in order to ensure that food products entering the market are of minimal risk to produce an infection.

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