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Antibiotic Resistance of *Escherichia coli* Isolated from Processing of Brewery Waste with the Addition of Bulking Agents

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Abstract: The aim of the study was to determine the drug resistance profile and to assess the presence of genes responsible for the production of extended-spectrum beta-lactamases in *Escherichia coli* isolated from energy-processed hop sediment with the addition of bulking agents. Antibiotic resistance was determined by the disk diffusion method and the PCR technique to detect genes determining the extended-spectrum beta-lactamases (ESBLs) mechanism. A total of 100 strains of *E. coli* were collected. The highest resistance was found to aztreonam, tetracycline, ampicillin, ticarcillin, and ceftazidime. The bacteria collected were most often resistant to even 10 antibiotics at the same time and 15 MDR strains were found. The ESBL mechanism was determined in 14 isolates. Among the studied genes responsible for beta-lactamase production, *blaTEM* was the most common (64%). The study revealed that the analysed material was colonised by multi-drug-resistant strains of *E. coli*, which pose a threat to public health. The obtained results encourage further studies to monitor the spread of drug resistance in *E. coli*.

Keywords: *Escherichia coli*; extended-spectrum beta-lactamases; brewing; hot trub; bulking agents

Citation: Wolny-Koładka, K.; Zdaniewicz, M. Antibiotic Resistance of *Escherichia coli* Isolated from Processing of Brewery Waste with the Addition of Bulking Agents. *Sustainability* **2021**, *13*, 10174. https://doi.org/10.3390/su131810174

Academic Editor: Helvi Heinonen-Tanski

Received: 24 August 2021
Accepted: 9 September 2021
Published: 11 September 2021

1. Introduction

Hot trub (HT) is a waste generated in breweries during beer production and is closely related to the use of hops [1]. Hot trub is formed when wort and hops are boiled in the brewing house. Currently, there are ways to partially manage it (e.g., as fertiliser for plants, animal feed, substrate for cosmetics and tranquillisers), but a large proportion of HT still ends up in landfills. Therefore, Wolny-Koładka et al. [2] attempted, in their study, to assess the possibility of using RDF (refuse-derived fuels) and UFMSW (undersize fraction from municipal solid waste) as bulking agents in bio-drying of hop sediments in order to use them for energy purposes. The bio-drying process used in the above-mentioned study decreased the number or completely eliminated some pathogens, while for *Escherichia coli* the effect was not entirely satisfactory. A significant decrease in the *E. coli* number was noted in the bio-dried material, but the decrease was not complete.

E. coli is a broad-spectrum bacterium in the environment, readily acquiring drug resistance genes and also closely associated with humans [3]. *E. coli*, which is a commensal, conditionally pathogenic bacterium, is constantly present both in the gastrointestinal tract of mammals and in the environment, e.g., in water and soil [4]. In addition, *E. coli* is abundant in municipal solid waste (MSW), including RDF and UFMSW [5,6]. Therefore, it can be assumed that strains isolated from such heterogeneous material will exhibit different drug resistance. This is extremely concerning, because currently, to the best of our knowledge, no literature data are available on the prevalence of drug-resistant *E. coli* in MSW, RDF, and UFMSW. All current studies are focused on analysing the presence

of drug-resistant *E. coli* in wastewater, sewage sludge, landfill leachate, and wastewater treatment plants [7–10].

Bacterial resistance to antibiotics is a growing and global problem; therefore, monitoring this phenomenon and understanding its molecular basis are crucial [11]. One of the most common resistance mechanisms in *E. coli* is the ability to produce extended-spectrum beta-lactamases (ESBLs) [12]. Strains with ESBL-resistance are extremely dangerous from the epidemiological point of view, because they are able to hydrolyse all penicillins, cephalosporins and monobactams. Additionally, they can exhibit cross-resistance to *trimethoprim/sulfamethoxazole* and quinolones [13]. ESBL-encoding genes spread rapidly, also between strains of different species, due to their presence on conjugation plasmids [14]. It is for this reason that ESBL-producing *E. coli* strains are highly virulent and resistant to antibiotics. Additionally, these isolates can donate genes of resistance to many commonly used antibiotics, making rational antibiotic therapy difficult, which leads to the development of cross-resistance to different classes of antibiotics simultaneously. This is why it is so important to understand and monitor drug resistance in these bacteria and to determine the presence of ESBL-producing strains [3].

The aim of the study was to determine the drug resistance profile of *E. coli* isolated from the bio-dried mixtures of HT (which is a difficult to manage waste in beer production) and bulking agents, i.e., RDF and UFMSW. The analyses allowed us to determine whether the material processed for energy purposes contains multi-drug resistant *E. coli* strains which may pose a threat to public health.

2. Material and Methods

2.1. Preparation of the Substrate

The materials used to isolate *E. coli* were the substrates obtained during previous research described in the study of Wolny-Koładka et al. [2]. In short, it can be stated that these were wastes (RDF and UFMSW) mixed in with HT from an industrial brewery, which is a waste in beer production. UFMSW and RDF were mixed with HT in such a way that HT accounted for 30% of wet mass and waste (UFMSW and RDF) for 70% of wet mass. Then, 30.3 ± 0.6 kg of the mixture were placed in bioreactors in the 6 trials:

- HT 100% treatment, air-flow rate: 10 $dm^3 \cdot min^{-1}$;
- HT 100% treatment, air-flow rate: 5 $dm^3 \cdot min^{-1}$;
- UFMSW 70 wt% + HT 30 wt%, air-flow rate: 10 $dm^3 \cdot min^{-1}$;
- UFMSW 70 wt% + HT 30 wt%, air-flow rate: 5 $dm^3 \cdot min^{-1}$;
- RDF 70 wt% + HT 30 wt%, air-flow rate: 10 $dm^3 \cdot min^{-1}$;
- RDF 70 wt% + HT 30 wt%, air-flow rate: 5 $dm^3 \cdot min^{-1}$.

The mixtures prepared in this way were bio-dried in bioreactors. The analyses were aimed at determining whether, as a result of HT bio-drying with bulking agents, i.e., RDF and UFMSW, it is possible to obtain an alternative fuel with beneficial energy properties and stable in terms of microbiological risks.

2.2. Isolation and Identification of E. coli

Bacteria were isolated by serial dilution method by Koch. A selective chromogenic medium–TBX agar (Tryptone Bile X-glucuronide agar, BTL, Poland) was used and the cultures were incubated at 44 °C for 24 h to eliminate the growth of associated microflora [3]. After the incubation, green and blue bacteria colonies were selected for identification. Initial species identification began with the preparation of microscope slides and staining them according to the Gram method. Colonies that were identified as Gram-negative bacilli in the microscope slide and grew as green-blue colonies on TBX agar were multiplied and submitted for further species identification using the MALDI-TOF MS technique (Bruker Daltonik, Germany). The MALDI-TOF MS species identification was carried out according to the methodology recommended by the manufacturer and guidelines contained in the studies of other authors [15–17].

2.3. Drug Resistance and ESBL Detection

The disk diffusion method recommended by the European Committee on Antimicrobial Susceptibility [18] was used to determine the antibiotic resistance of the collected *E. coli* isolates; for antibiotics outside the list, we used the guidelines in the studies of other authors, i.e., Kronvall et al. [19]: cefalotin; Turnidge [20]: cefazolin; Barry et al. [21]: cefamandole; Sader et al. [22]: tetracycline. MHA medium (Mueller–Hinton agar, BTL, Poland) was poured quantitatively (15 mL) into sterile Petri dishes. Then, from 18–24 h clean *E. coli* culture, single bacterial colonies were collected with a sterile swab and placed in test tubes with saline (0.9% NaCl), vortexed and adjusted to a concentration of 0.5 McFarland using a densitometer (DEN-1, Biosan, Poland). The sterile swab was immersed in the suspension and the inoculum was evenly spread over the previously prepared Petri dishes with MHA medium. Sterile antibiotic disks (Oxoid, Ireland) were applied to the obtained cultures. The ESBL mechanism was detected with the double-disk synergy test [23]. After incubation for 18–24 h at 37 °C, the growth inhibition diameters around the antimicrobial disks were measured (mm) and the results were compared with the breakpoint values recommended by the EUCAST [18]. The reference strain of *E. coli* ATCC 25922 was used as a quality control for the diffusion disk method used.

2.4. DNA Extraction and Detection of ESBL-Determining Genes

Bacterial genomic DNA was extracted from the cultures obtained in the study and from the control *E. coli* strain ATCC 25922 using the Genomic Mini DNA extraction kit (A&A Biotechnology, Poland), following the manufacturer's instructions. To evaluate the presence of ESBL-determining genes, PCR tests were conducted using specific primers (Table 1): *blaCTXM*-3 [24], *blaCTXM*-9 [25], *blaOXA*, *blaSHV* and *blaTEM* [26].The reactions were performed in 25 µL containing 50 ng of DNA template, 12.5 pM of each primer, 2.5 mM of dNTP, 1× PCR buffer, and 1 U Dream-Taq DNA polymerase (Thermo Scientific, US). The following temperature profile was used for the reactions: initial denaturation at 95 °C for 5 min, followed by 35 cycles at 94 °C for 45 s, annealing for 45 s at temperatures corresponding to individual primers, then extension at 72 °C for 1 min with a final extension at 72 °C for 10 min, followed by storage at 4 °C. The PCR amplifications were performed in T100 Thermal Cycler (Bio-Rad, USA). The PCR products were electrophoresed for 60 min in 1× TBE, 1% agarose gel, stained with Simply Safe (0.5 mg/mL; EurX, Poland), visualised in UV light and documented using the Gel Doc system (Bio-Rad, US).

Table 1. Description of primers used in the study.

Gene	5′-3′ Sequence	Annealing Temperature (°C)	Product Length (bp)	Reference
blaCTXM-3	F: GTTACAATGTGTGAGAAGCAG R: CCGTTTCCGCTATTACAAAC	60	800	[24]
blaCTXM-9	F: GTGACAAAGAGAGTGCAACGG R: ATGATTCTCGCCGCTGAAGCC	54	860	[25]
blaOXA	F: ACACAATACATATCAACTTCGC R: AGTGTGTTTAGAATGGTGATC	61	813	[26]
blaSHV	F: CACTCAAGGATGTATTGTG R: TTAGCGTTGCCAGTGCTCG	52	885	[26]
blaTEM	F: ATTCTTGAAGACGAAAGGGC R: ACGCTCAGTGGAACGAAAAC	60	1150	[26]

3. Results and Discussion

3.1. E. coli Drug Resistance Profile

In total, 100 *E. coli* isolates were collected. There was no difference in the number of *E. coli* isolates among all six trials. Therefore, the strains for drug resistance analyses were selected in a representative manner from all six trials. Disk diffusion tests allowed for determining the antibiotic resistance of bacteria, as shown in Table 2. The most common

were resistance to aztreonam, tetracycline, ampicillin, ticarcillin, and ceftazidime. In contrast, no resistance to ciprofloxacin and piperacillin/tazobactam were observed. *E. coli* exhibited resistance to a maximum of 10 antibiotics at the same time, with 11 isolates sensitive to all tested antibiotics. Moges et al. [27] found 100% resistance to ampicillin and 38% resistance to tetracycline in *E. coli* isolated from hospital wastewater; they also found high resistance to cephalothin (23%), trimethoprim/sulfamethoxazole (38%), and cefotaxime (23%). In *E. coli*-contaminated water in Ghana, Odonkor et al. [28] determined the bacteria resistance to tetracycline (21.45%), trimethoprim/sulfamethoxazole (18.56%), ampicillin (11.32%), ciprofloxacin (8.25%), amikacin (7.22%), gentamicin (5.15%), and cefotaxime (4.12%).

Table 2. Antibiotic resistance of *E. coli*.

Antibiotic (Symbol, µg)	Limit Values (mm)	Number of Isolates *n* = 100
Amikacin (AK, 30)	18 [18]	5
Amoxicillin / Clavulanic acid (AMC, 30) *	19 [18]	6
Ampicillin (AMP, 10)	14 [18]	**23**
Aztreonam (ATM, 30)	26/21 [18]	**30**
Cefamandole (MA, 30)	18/14 [21]	4
Cefepime (FEP, 30)	27/24 [18]	2
Cefotaxime (CTX, 30) *	20/17 [18]	5
Cefoxitin (FOX, 30)	19 [18]	8
Ceftazidime (CAZ, 30) *	22/19 [18]	**21**
Cefalotin (KF, 30)	13 [19]	10
Cefazolin (KZ, 30)	23/19 [20]	5
Ciprofloxacin (CIP, 5)	25/22 [18]	0
Gentamicin (CN, 10)	17 [18]	7
Netilmicin (NET, 30)	15/12 [18]	6
Piperacillin (PRL, 100)	20/17 [18]	6
Piperacillin/Tazobactam (TZP, 110)	20/17 [18]	0
Tetracycline (TE, 30)	15/11 [22]	**25**
Ticarcillin (TIC, 75)	23/20 [18]	**22**
Tobramycin (TOB, 10)	16 [18]	8
Trimethoprim/Sulfamethoxazole (SXT, 25)	14/11 [18]	11
ESBL	-	14
blaTEM	-	**64**
blaCTXM-3	-	**25**
blaCTXM-9	-	**23**

* Antibiotics used to detect the ESBL mechanism, >20 values are in bold.

15 MDR (multi-drug-resistant) strains were found, which are described in the literature as having resistance to antibiotics of three or more classes [29]. The 15% presence of MDR strains can be considered low when compared to the analysis of Odonkor et al. [28] who detected 49.48% of MDR *E. coli* in drinking water in Ghana. Additionally, a high prevalence of these isolates was found by Moges et al. [27]: 81.5% and 54.2%, respectively, for hospital and non-hospital wastewater. These studies clearly show that the strain isolation site plays a key role in *E. coli* drug resistance.

3.2. ESBL-Determining Genes

Among the studied genes responsible for extended spectrum beta-lactamase production in *E. coli*, *blaTEM*, *blaCTXM-3* and *blaCTXM*-9 were found, and there were no *blaOXA* and *blaSHV* (Table 3). In addition, significant differences were observed in the frequency of both genes. The *blaTEM* (64%) gene was clearly dominant. This was similar to the study of Adefisoye and Okoh [30] carried out in South Africa, in which *blaTEM* in wastewater-isolated *E. coli* reached 56.4%. The determined levels of *blaCTXM*-3 (25%) and *blaCTXM*-9 (23%) were similar and, in all cases, these genes were found together

with *blaTEM* (Table 3). According to Bradford [31], among all genes responsible for beta-lactamase formation, the TEM family is most often detected in Gram-negative bacteria. Furthermore, the TEM and SHV gene families are most frequently detected right in *E. coli* and *K. pneumoniae* [31]. Baraniak [32] argued that *blaTEM* genes are extremely important virulence factors in *E. coli*, since, due to the diversity of produced enzymes, they enable the adaptation of bacteria to different environments and enhance the resistance of these strains to beta-lactam antibiotics. The high ampicillin resistance described in our study is correlated with the presence of *blaTEM* [11]. According to Brinas et al. [33], *blaTEM* genes are common in ampicillin-resistant *E. coli* strains isolated from both animals and humans.

The ability to produce extended-spectrum beta-lactamases was found for 14 isolates. However, according to Hanberger et al. [34], the prevalence of human *E. coli* strains with the ESBL phenotype in Europe is 3.90% and varies considerably between countries. Among the 14 isolates in which the ESBL mechanism was detected using the disk diffusion method, 8 did not have any of the studied genes. This may be due to the fact that resistance mechanisms are conditioned multigenically [3,35]. On the other hand, despite the presence of at least one of the studied genes determining extended-spectrum beta-lactamase production, the ESBL mechanism was not presented phenotypically in 56 isolates. Similar observations were described in the study of Wolny-Koładka and Lenart-Boroń [35], where 38% of *E. coli* had ESBL-determining genes, and the mechanism was not revealed in phenotypic tests. Therefore, despite the discrepancy in results between the disk diffusion test and the PCR test, it is reasonable to detect this mechanism both as part of the prepared antibiogram and to use genetic methods. This is extremely important epidemiologically and will facilitate the monitoring and control of potential infections [3,36]. The results obtained in this study support the principle that the correlation between phenotypic and genetic test results is low. This may be due to the fact that drug resistance is multigene-conditioned or genes, although present in *E. coli*, were not expressed, which could have been observed in drug resistance analysis [3,35].

Table 3. Phenotypical and genotypical profiles of *E. coli*, including antibiotic resistance and ESBL genes.

Isolate No.	Antibiotic Resistances	ESBL Genes
1.	AMC, AMP, ATM, CTX, FOX, CAZ, KZ, TIC, SXT	*blaTEM, blaCTXM-3, blaCTXM-9*
2.	KF, SXT	*blaTEM, blaCTXM-3, blaCTXM-9*
3.	CAZ, TIC	
4.	AMP, ATM, KZ, CN, TIC	*blaTEM, blaCTXM-3*
5.	CTX, CAZ, TIC, SXT	*blaTEM, blaCTXM-3*
6.	AMC, AMP, ATM, KZ, TE, TIC	*blaTEM, blaCTXM-9*
7.	KF	*blaTEM, blaCTXM-3*
8.	KZ, TIC	
9.	CN	
10.	AMP, ATM, TE, TIC	*blaTEM, blaCTXM-3, blaCTXM-9*
11.	KZ	*blaTEM, blaCTXM-9*
12.	CAZ	
13.	CN	
14.	KF	*blaTEM, blaCTXM-3*
15.	AMP, ATM	*blaTEM*
16.		*blaTEM, blaCTXM-3*
17.	CAZ, KF, TIC	*blaTEM*
18.	TOB	*blaTEM, blaCTXM-3*
19.	AMP	
20.	AMP, ATM, TIC	*blaTEM*
21.	ATM, SXT	*blaTEM*
22.	TE, TIC	*blaTEM, blaCTXM-3*

Table 3. *Cont.*

Isolate No.	Antibiotic Resistances	ESBL Genes
23.	CAZ, TE	*blaTEM*
24.	AMC, AMP, ATM, MA, CTX, FOX, TE, TIC	*blaTEM*
25.	TE	*blaTEM*
26.	ATM, FOX, TE, TIC	*blaTEM, blaCTXM-3*
27.	KF	
28.	TE	*blaTEM*
29.	AMP, ATM	*blaTEM*
30.	AMP	
31.	AMP, ATM	
32.	MA	*blaTEM, blaCTXM-9*
33.	KF	*blaTEM*
34.	CTX	
35.	AMP, ATM, FOX, TE	*blaTEM, blaCTXM-3*
36.	MA	*blaTEM*
37.	AMP, ATM, TE	*blaTEM, blaCTXM-3, blaCTXM-9*
38.	CAZ	*blaTEM*
39.	ATM	*blaTEM, blaCTXM-9*
40.	ATM	
41.	NET	*blaTEM*
42.	AMP, ATM	*blaTEM, blaCTXM-3, blaCTXM-9*
43.	TE	*blaTEM*
44.	AM, ATM, CTX, FOX, KF, NET, TE, TIC, TOB, SXT	*blaTEM, blaCTXM-3*
45.		*blaTEM, blaCTXM-9*
46.	FEP	
47.	SXT	*blaTEM*
48.	ATM	
49.	ATM, CAZ, TE, TIC, TOB	*blaTEM, blaCTXM-9*
50.		
51.	PRL, SXT	*blaTEM, blaCTXM-9*
52.	AMP, TIC	
53.	CN, TE, TOB	*blaTEM, blaCTXM-3*
54.	ATM	
55.	AMC, CAZ, KF, NET, TE, TIC	*blaTEM, blaCTXM-9*
56.	TE	
57.	PRL	
58.	ATM, CAZ	*blaTEM, blaCTXM-9*
59.	CAZ	
60.	CN	*blaTEM, blaCTXM-9*
61.	AMP, TE, TIC	*blaTEM, blaCTXM-9*
62.	CAZ	
63.	ATM	*blaTEM*
64.		
65.	CAZ	*blaTEM, blaCTXM-3*
66.	AMP, TIC, TOB	
67.		*blaTEM*
68.	CN, TOB	*blaTEM*
69.		
70.	AMC, AMP, NET, TE, TIC	*blaTEM, blaCTXM-3*
71.		
72.	CAZ	
73.	ATM	
74.	ATM, CAZ, TE	
75.	ATM	
76.		*blaTEM, blaCTXM-3*
77.	ATM	*blaTEM, blaCTXM-3*
78.	PRL, TE	

Table 3. *Cont.*

Isolate No.	Antibiotic Resistances	ESBL Genes
79.	TE	
80.	AMP, ATM, TIC, SXT	*blaTEM*
81.	TE	
82.		*blaTEM*
83.	KF, SXT	*blaTEM, blaCTXM-3, blaCTXM-9*
84.		*blaTEM*
85.	PRL, TOB	*blaTEM, blaCTXM-9*
86.	AMP, ATM, CAZ, KF, CN, TIC	*blaTEM*
87.	NET, PRL	*blaTEM, blaCTXM-3*
88.	TIC	
89.	CAZ, SXT	*blaTEM*
90.		*blaTEM, blaCTXM-9*
91.	AMP, ATM, FOX, CAZ, PRL, TOB	*blaTEM, blaCTXM-9*
92.	TE	*blaTEM, blaCTXM-3*
93.	TE	*blaTEM, blaCTXM-3, blaCTXM-9*
94.	ATM, FOX, CAZ	*blaTEM, blaCTXM-3, blaCTXM-9*
95.	FEP	
96.	TE	*blaTEM*
97.	AMP, CAZ	
98.	NET	*blaTEM, blaCTXM-9*
99.	MA, CAZ	
100.	AMP, FOX, SXT	

4. Conclusions

The study allowed for the isolation, identification and evaluation of the drug resistance profile of 100 *E. coli* strains originating from processed HT with the addition of bulking agents, i.e., RDF and UFMSW. Among the collected strains, many were resistant to the tested antibiotics, and the bacteria with the MDR phenotype was found. The *blaTEM* gene was most common of all genes responsible for the production of extended-spectrum beta-lactamases. The results obtained in this study provide new and unique knowledge on the presence of multi-drug resistant *E. coli* strains in substrates processed for energy purposes. From the epidemiological point of view, *E. coli* strains inhabiting the analysed materials are dangerous for people working at their storage and processing. Additionally, as a result of their long-term storage, drug-resistant *E. coli* can spread outside the landfill area (e.g., through landfill leachate, air currents, wild animals). The collected results clearly demonstrate the need for further studies to assess the presence of drug-resistant bacteria in municipal waste. On this basis, it will be possible not only to assess the presence of epidemiological hazards related to waste management, but also to look for links between, for example, the type and origin of stored waste and the degree of its colonisation by drug-resistant microorganisms.

Author Contributions: Conceptualization, K.W.-K.; methodology, K.W.-K.; software, K.W.-K.; validation, K.W.-K., M.Z.; formal analysis, K.W.-K.; investigation, K.W.-K.; resources, M.Z.; data curation, K.W.-K.; writing—original draft preparation, K.W.-K.; writing—review and editing, K.W.-K.; visualization, K.W.-K.; supervision, K.W.-K. and M.Z.; project administration, M.Z.; funding acquisition, M.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Centre for Research and Development, Poland (Grant No. LIDER/ 46/0185/L-9/17/NCBR/2018).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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