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Antibiotic Resistance in Wastewater: Transmission Risks for Employees and Residents around Wastewater Treatment Plants

vorgelegt von:

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Affidavit

I, Daloha Rodriguez Molina, hereby declare that the submitted thesis entitled

Antibiotic Resistance in Wastewater: Transmission Risks for Employees and Residents around Wastewater Treatment Plants

is my own work. I have only used the sources indicated and have not made unauthorised use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

I further declare that the submitted thesis or parts thereof have not been presented as part of an examination degree to any other university.

Munich, 20. October 2022

Daloha Rodriguez Molina

Confirmation of congruency

I, Daloha Rodriguez Molina, hereby declare that the electronic version of the submitted thesis entitled

Antibiotic Resistance in Wastewater: Transmission Risks for Employees and Residents around Wastewater Treatment Plants

is congruent with the printed version both in content and format.

Munich, 20. October 2022

Daloha Rodriguez Molina

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List of abbreviations

AR	Antibiotic resistance
AWARE	Antibiotic Resistance in Wastewater: Transmission Risks for Employees and Residents around Wastewater Treatment Plants
CI	Confidence interval
DAG	Directed acyclic graph
E. coli	*Escherichia coli*
ESBL	Extended-spectrum beta-lactamase
IPSW	Inverse probability of sample weights
MALDI-TOF MS	Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry
MAR	Missing at random
OR	Odds ratio
TAC	Thesis advisory committee
TBX	Tryptone Bile X-Glucuronide
WWTP	Wastewater treatment plants

List of publications

Publication list

1. **Rodríguez-Molina D**, Berglund F, Blaak H, Flach CF, Kemper M, Marutescu L, Gradisteanu GP, Popa M, Spießberger B, Weinmann T, Wengenroth L, Chifiriuc MC, Larsson DGJ, Nowak D, Radon K, de Roda Husman AM, Wieser A, Schmitt H. Carriage of ESBL-producing Enterobacterales in wastewater treatment plant workers and surrounding residents - the AWARE Study. Eur J Clin Microbiol Infect Dis. 2021 Dec 13:1–16. doi: 10.1007/s10096-021-04387-z. Epub ahead of print.

2. **Rodríguez-Molina D**, Berglund F, Blaak H, Flach CF, Kemper M, Marutescu L, Gradisteanu GP, Popa M, Spießberger B, Wengenroth L, Chifiriuc MC, Larsson DGJ, Nowak D, Radon K, de Roda Husman AM, Wieser A, Schmitt H. International travel as a risk factor for carriage of extended-spectrum β-lactamase-producing *Escherichia coli* in a large sample of European individuals - The AWARE Study. Int J Environ Res Public Health. 2022, 19, 4758. https://doi.org/10.3390/ijerph19084758.

Appendix list

1. Wengenroth L, Berglund F, Blaak H, Chifiriuc MC, Flach CF, Pircalabioru GG, Larsson DGJ, Marutescu L, van Passel MWJ, Popa M, Radon K, de Roda Husman AM, **Rodríguez-Molina D**, Weinmann T, Wieser A, Schmitt H. Antibiotic Resistance in Wastewater Treatment Plants and Transmission Risks for Employees and Residents: The Concept of the AWARE Study. Antibiotics. 2021 Apr 21;10(5):478. doi: 10.3390/antibiotics10050478.

1. Contributions to the Individual Publications

1.1 Contribution to publications 1 and 2

Publication 1: Carriage of ESBL-producing Enterobacterales in wastewater treatment plant workers and surrounding residents - the AWARE Study

Publication 2: International travel as a risk factor for carriage of extended-spectrum β-lactamase-producing *Escherichia coli* in a large sample of European individuals - The AWARE Study

For these publications, I participated in all steps of this project, with the exception of microbiology analyses. Then, with the help of my supervisor, Prof. Dr. Radon, and the rest of the AWARE (Antibiotic Resistance in Wastewater: Transmission Risks for Employees and Residents around Wastewater Treatment Plants) Consortium, I was in charge of the conceptual design and characterizing the research question for this article. I performed all data-related steps, including data cleaning, wrangling, merging, validation, and analysis. I applied multiple imputation for handling missing data and inverse probability of sample weights (IPSW) to correct for low participation response. I ran all exploration and inference analyses. I drew the directed acyclic graph (DAG) and checked for causal inference assumptions (only for publication 1). I generated all tables and figures for the publications. With the help of the AWARE team and my thesis advisory committee (TAC), I interpreted the data. Finally, I wrote the first draft of each paper, coordinated discussions with project partners, made necessary changes as per comments from co-authors before submission, submitted the manuscripts to a pre-print server (medRxiv) and to the respective journals for peer review, and handled all correspondence with the journal, including response to peer reviewers.

1.2 Contribution to Appendix 1

Appendix 1: Antibiotic Resistance in Wastewater Treatment Plants and Transmission Risks for Employees and Residents: The Concept of the AWARE Study

This paper outlines the scientific rationale, main and specific aims, methodological procedures, and expected outcomes of the AWARE project. I have been working with the AWARE project since its inception in 2016-2017. I was in charge of drafting the study protocol, obtaining ethical clearance, designing and piloting the study questionnaires, supervising the translation and back-translation of questionnaires from English into German, designing and maintaining the project webpage (www.aware-study.eu) as well as designing all the corporate image of the project (logo, newsletter template, slides template), piloting air and water sample methods in Germany, establishing and piloting an online data collection system on LimeSurvey, managing pilot data, generating pilot data analysis reports, and deciding along with the AWARE Consortium on changes needed for the questionnaire and sample collection methods after the pilot study. All of these activities were summarized and incorporated into this publication. After the initial draft

written by Dr. Laura Wengenroth, I was part of the co-authoring team that revised, suggested changes, and approved the final published manuscript.

2. Introductory summary

Modern medicine and public health must face many challenges during this century. One of such main challenges is antibiotic resistance (AR). Although AR is an ancient evolutionary response from bacteria to pressures in their environment that confers them the *intrinsic* ability to be immune to antibiotics, the surge of *acquired* AR in the last decades has posed a challenge to modern medicine (1). The production of Extended-Spectrum Beta-Lactamases (ESBLs) is one of the described mechanisms for acquired AR. ESBLs are one type of resistance enzymes that grants bacteria the ability to inhibit commonly used antibiotics by breaking the beta-lactam ring in their chemical structure (2,3). Therefore, the antibiotic is no longer active in killing the bacteria. Some of the antibiotic families against which ESBL-producing bacteria are resistant include penicillins, cephalosporins and monobactams (1,2,4). These antibiotics are widely prescribed to treat infections caused by many bacteria, including *Escherichia coli*. This species of bacteria is part of the normal gut microflora in humans, and poses a risk for microbial infections either when out of its usual physiological environment, i.e. causing urinary tract infections, skin infections, and other types of extra-intestinal infections, or when producing enterotoxins that lead to gastrointestinal disorders (5).

Non-pathogenic *E. coli* can also acquire antibiotic resistance to a wide range of beta-lactams. When this happens, and the mechanism of resistance is through the synthesis of ESBLs, it is denominated ESBL-producing *E. coli* (5). Human beings can carry ESBL-producing *E. coli* in their gut microbiota without realizing it. Although there are no immediate adverse health consequences to carrying ESBL-producing *E. coli* in the gut microbiota, this and other types of AR bacteria represent a therapeutic challenge to healthcare providers when disease strikes (6). Treatment options thus become more limited: last-resort antibiotics might be needed to treat common infections, which increases the cost of healthcare, the average length-of-stay during hospitalizations, the risk of adverse effects, and the risk of mortality (7–9). The current literature reports a dramatic increase in the prevalence of ESBL-producing E. coli in the last decades. In 2003-2005 the mean global estimate was 2.6% while in 2015-2018 it was 21.1% (10).

Enteric bacteria such as ESBL-producing *E. coli* coming from human beings and other sources are regularly excreted into the sewage (11). Other sources shedding ESBL-producing *E. coli* into sewage water include farming and husbandry activities as well as hospitals (12). Apart from that, antibiotics are also commonly excreted into the sewage system from human consumption and excretion, pharmaceutical companies, or veterinary and farming activities (8,13–24). Sewage water is then processed in wastewater treatment plants (WWTPs) until the treated water is free from pathogens and therefore safe to shed into the water environment, i.e. rivers, lakes, etc. Although reduction in the amount of antibiotics and AR bacteria has been reported through the wastewater treatment process, inactivating, eliminating, or reducing the amount of AR resistant bacteria such as ESBL-producing *E. coli* is not the main objective of WWTPs (25). Further, because sewage water is collected from different sources deriving from all kinds of human activities, WWTPs serve as unintentional collection points for antibiotics and AR bacteria and as hotspots for the dissemination of clinically relevant resistant bacteria into the water environment

(25). AR bacteria has been found in several water reservoirs such as recreational waters (26–32) and water for agricultural irrigation (22,33–35), as well as in water within the WWTP process, including at WWTP locations such as influent, effluent, aeration tank, and sludge facilities (12,24,36–39).

By defining ingestion of droplets, hand-to-mouth contact, and inhalation of droplets of wastewater contaminated with AR bacteria as potential exposure routes (40–43), and considering that ESBL-producing *E. coli* has been found up to 150 meters upwind and downwind from animal farms (44), water collected and processed at WWTPs as well as aerosols coming from the plants and carrying ESBL-producing *E. coli* could then pose a risk for WWTPs workers or residents living in close proximity to WWTPs. The presence of AR bacteria in water (23,40,41,45–48) and air samples (42,43,49) has been widely reported. Nevertheless, the AWARE (Antibiotic Resistance in Wastewater: Transmission Risks for Employees and Residents around Wastewater Treatment Plants) Study is unique in its kind, as it is the first study -within our knowledge- aiming at characterizing the transmission risks of AR bacteria to employees working at WWTP as well as to people living near a local WWTP.

Nevertheless, WWTPs are only one of many potential environmental sources of AR bacteria exposure in humans (50). Agriculture, animal husbandry and healthcare are also environmental sources of AR bacteria, specifically because of the use of antibiotics (6,8,33,51–55). The carriage of ESBL-producing *E. coli* in the human gut microbiota has been associated with a myriad of potential personal risks factors including the use of antibiotics (56–58), travels to high-risk areas for AR (7,56,57,59–76), consuming food contaminated with AR bacteria (77,78), working at animal markets, farms, slaughterhouses, dairy or healthcare facilities (79–94). One of the issues with the current state of the literature is that these factors have been widely described and characterized in studies focused on specific study populations that are already at a high risk of carrying ESBL-producing bacteria such as travellers (57,60,62,66,70,72,75,95), patients and healthcare workers (88,93,94,96–98), swimmers (99–101), farmers (81–84,86,87,89,91,92), and slaughterhouse workers (80). Another issue is that studies tend to rely on a small convenience sample of participants such as students (7,56,68,69). Therefore, the aims of this doctoral dissertation were:

1. To find out if WWTP workers and people living close to a WWTP are at higher risk of carrying ESBL-producing E. coli in the gut microbiota, in comparison to the general population
2. To identify risk factors for carrying ESBL-producing E. coli in a sample of individuals recruited from the general population in Romania, Germany and the Netherlands.

Secondary aims include:

1. To find out what is the current estimated prevalence of ESBL-producing E. coli in the three studied countries
2. To estimate the average prevalence of ESBL-producing *E. coli* in each of the study groups: WWTP workers, nearby residents, and distant residents

3. To identify the most important risk factor(s) for ESBL-producing *E. coli* in a subset of participants stemming from the general population in each of the three countries.

In order to answer these research questions, I carried out my research within the scope of the AWARE study. This doctoral dissertation addresses a subset of the main aims of the AWARE study. Appendix 1 (102) lists all the relevant research aims of the AWARE study.

2.1 The AWARE Study

In order to explore and characterize the transmission risks of antibiotic resistant bacteria and bacterial resistance genes from WWTPs to workers within the plants and residents living in close proximity to such plants, we designed a cross-sectional study with data collected in three European countries: Germany, the Netherlands, and Romania. The AWARE Consortium is made up of researchers from these three countries, in addition to Sweden. We adapted our recruitment methods to each of the three countries and the full methodology can be found in the Appendix 1 (102). A summary of the methodology used is described as follows (Fig 1).

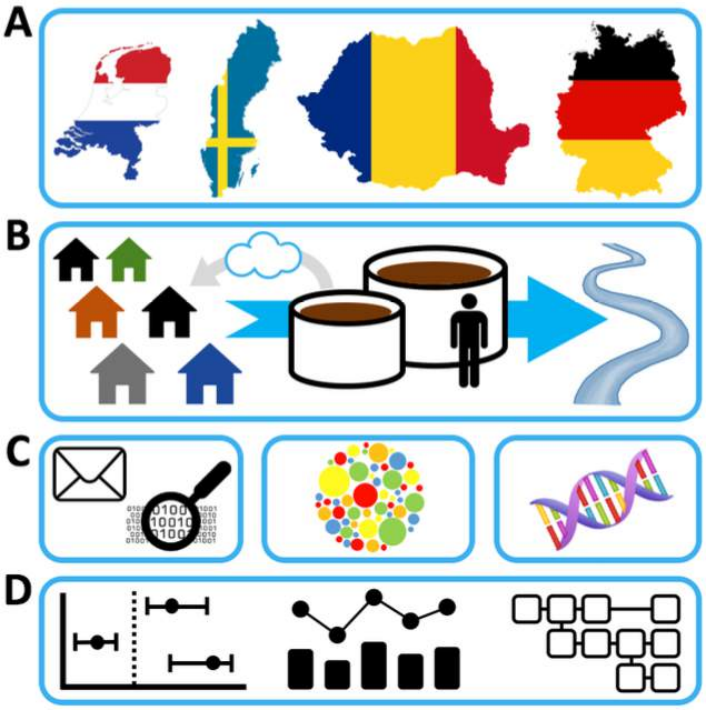

Fig 1. Overview of the AWARE Study. Source: AWARE study proposal. The image shows A) the four participating countries: The Netherlands, Sweden, Romania and Germany; B) A summary of the study sites and population: WWTP workers and residents living nearby (≤ 300 meters) a local WWTP potentially at an elevated risk of exposure to antibiotic-resistant bacteria from the WWTP, in comparison to an unexposed group of residents living more than 1000 meters away from any WWTP; C) An illustration of the epidemiological, microbiological, and metagenomics methods used; D) The desired outcomes: an epidemiological evaluation of the prevalence of antibiotic-resistant bacteria, especially ESBL-producing *E. coli* in stool samples of participants, and

determining if the exposed population has elevated odds for carriage of ESBL-producing *E. coli* in comparison to the unexposed population.

In the Netherlands, WWTPs are administered by waterboards, which are regional water authorities. The recruitment unit in Germany and Romania was the WWTP, while in the Netherlands it was the waterboards. Because we had to collect water samples from each plant, and in order to adequately preserve them during sample transportation until they arrived at the processing laboratories, eligibility criteria for WWTPs included being located within a 4-hour drive to the local laboratory, and to have the largest amount possible of both WWTP workers and nearby residents so that we could reach the desired sample size as quick as possible. We designed a sampling frame of all potential WWTPs and ordered them in increasing order of distance to the lab and in decreasing order of the number of WWTP workers and nearby residents. We then recruited WWTP following this order. In the Netherlands, all waterboards in the country were addressed, independently of distance or number of WWTP workers and residents. In each of the countries where data collection was to be carried out, we invited the WWTP operators, directors, or competent authority in deciding whether the WWTP was to participate in the study. Once the WWTP had been recruited, we proceeded to invite participants. Because WWTP workers are of working age, and in order to make our groups comparable, we restricted the age of participation to be between 16 and 67 years of age.

We defined two exposed groups: WWTP workers and residents living closer than 300 meters away from WWTPs (from now on called nearby residents). We also defined a comparison group of residents living more than 1000 meters away from WWTPs. We invited WWTP workers through the WWTP or waterboard where they worked, by presenting the project at their workplace and by sending personalized paper invitation letters to the WWTP or electronic ones via email. To identify eligible residents living closer to each of the recruited WWTP, we used Google Maps™ to draw a 300-meter radius around each WWTP. All households within that range were eligible. We identified the streets where these households were located. In Germany, we asked the local civil registries to provide us with the name and age of residents registered in these households. Then, we sent personalized invitation letters to each eligible participant living in these households. A similar approach was used for distant residents, but with households more than 1000 meters away from the local WWTP. In Romania, and in German locations where collecting address information of each eligible participant was not possible, we employed door-to-door strategies: In Romania, the data collection team visited each household, while in Germany the team went door to door depositing impersonal invitation letters to all residents of each household that were between 16 and 67 years of age. In the Netherlands, only distant residents were recruited.

Assuming that the background average prevalence of ESBL-producing *E. coli* in the general population across the three countries was of 8%, and aiming at detecting a minimum odds ratio (OR) of 1.7 between both WWTP workers and the comparison group as well as between nearby residents and the comparison group, at a 5% statistical significance level and with 80% statistical power, we arrived at a minimum desired sample size of 150 workers per country (450 workers in

total), 800 nearby residents (400 in Germany and 400 in Romania), and 1200 distant residents (400 in each of the three countries).

Table 1. Summary of the recruitment process in the AWARE Study.

		Germany	The Netherlands	Romania
OVERALL	Invited	4743	14544	1147
	Enrolled	631	1287	749
	Excluded	151	461	115
	Final sample	480	826	634
	Response	10%	6%	55%
WWTP WORKERS	Invited	137	626	247
	Enrolled	58	207	163
	Excluded	28	46	10
	Final sample	30	161	153
	Response	22%	26%	62%
NEARBY RESIDENTS	Invited	1453	Nearby residents were not recruited in The Netherlands	620
	Enrolled	156		413
	Excluded	55		83
	Final sample	101		330
	Response	7%		53%
DISTANT RESIDENTS	Invited	3153	13918	280
	Enrolled	417	1080	173
	Excluded	68	415	22
	Final sample	349	665	151
	Response	11%	5%	54%

Each enrolled participant was asked to fill out an online questionnaire including questions about sociodemographics and personal risk factors for AR including job history, contact with animals, contact with patients or human tissues at work, travel information, health information (use of antibiotics and antacids, history of surgeries and hospitalization, personal history of diarrhea, respiratory health, and self-reported health status). We also asked WWTP workers about their occupational tasks, the use of personal protective equipment, and the areas of the WWTP where they most frequently carried out their tasks. Finally, operators were asked to additionally fill out a questionnaire exploring the operational characteristics of the WWTP they were leading. All explored personal variables were restricted to the last 12 months. We developed the questionnaires using a combination of expert opinion from within the AWARE Consortium and previously validated questions whenever possible. The initial language for developing questionnaires was English. Afterwards, we translated and back-translated the questionnaires into the local languages. Additional recruitment methods in Germany included a recruitment campaign on Facebook and articles about the AWARE study published in local newspapers. We tried increasing the participation response by sending participants a written reminder one and three weeks after the first invitation letter. Finally, we also used incentives: in Germany, all participants who completed all steps of data collection were eligible to winning Amazon vouchers

in a raffle, and in the Netherlands all participants who successfully completed data collection received an amazon voucher of 20 EUR. Despite our attempts, response was low in Germany and the Netherlands (Table 1).

We also collected one stool sample from each participant. Stool samples allowed us to directly study the outcome of interest: the presence of ESBL-producing *E. coli*. Within 24 hours of sampling, samples were collected and transported to the laboratory under refrigerated conditions (between 2 ºC and 8 ºC). Either Tryptone Bile X-Glucuronide (TBX, used in the Netherlands and in Romania) or MacConkey agar (in Germany) were used as positive controls. This means that each sample was inoculated into these discs and the sample had to produce a positive culture for the desired bacteria. Otherwise, the sample was discarded. To evaluate the presence of ESBL-producing *E. coli*, we inoculated the samples into ChromID® ESBL. All samples were incubated at 36 ºC ± 1 ºC for 24-48 hours. If the culture was positive for the bacteria of interest, we took two separate isolates and ran a phenotypic confirmation. Last, we used a spectrometry technique called MALDI-TOF MS for species identification.

2.2 Publication 1: Carriage of ESBL-producing Enterobacterales in wastewater treatment plant workers and surrounding residents - the AWARE Study

The main aim of the analyses carried out in this publication (103) was to determine whether WWTP workers and nearby residents were more likely to be carriers of ESBL-producing *E. coli* and other Enterobacterales in their gut microbiota, in comparison to distant residents. To properly characterize the WWTP as the main source of exposure, we excluded participants who worked in healthcare, at farms or at slaughterhouses. The total sample size consisted of 1940 participants: 826 from the Netherlands, 634 from Romania, and 480 from Germany. (Table 2).

According to our data, the prevalence of ESBL-producing E. coli in the three countries was 13%. The prevalence per country was 6% in the Netherlands, 7% in Germany, and 28% in Romania.. In stratified logistic regression analyses per country, adjusting for potential confounders, and in comparison to distant residents, the adjusted Odds Ratio (OR) and corresponding 95% confidence interval (CI) for carriage of ESBL-producing *E. coli* among workers in the Netherlands was 0.95 (0.37–2.44) and in Romania was 2.34 (1.22–4.50). In Germany, the model did not converge due to lack of variability (all workers were negative for ESBL-producing *E. coli* in the stool sample), so this parameter could not be estimated. For nearby residents, the adjusted OR and corresponding 95% CI in Germany was 0.81 (0.29–2.30) and in Romania was 3.17 (1.80–5.59). Therefore, we found that both WWTP workers and nearby residents in Romania were more likely to carry ESBL-producing *E. coli* in their gut microbiota than distant residents.

Table 2. Descriptive characteristics of the studied population by country and participation group, n = 1940, AWARE Study, 2021

			Germany					The Netherlands[a]				Romania				
Variable	**Missings**	**Overall**	**Overall**	**WWTP worker**	**Nearby resident[c]**	**Distant resident[b]**	**p**	**Overall**	**WWTP worker**	**Distant resident [b]**	**p**	**Overall**	**WWTP worker**	**Nearby resident [c]**	**Distant resident [b]**	**p**
n		1940	480	30	101	349		826	161	665		634	153	330	151	
Age, years (median [IQR])	0	49 [36, 58]	47 [35, 57]	52 [44, 55]	48 [35, 58]	45 [34, 56]	0.161	54 [40, 61]	54 [45, 59]	55 [39, 61]	0.710	43 [34, 53]	49 [41, 53]	41 [32, 54]	40 [33, 50]	**<0.001**
Sex, n (%) = Male	4	938 (48)	211 (44)	24 (80)	51 (50)	136 (39)	**<0.001**	403 (49)	150 (93)	253 (38)	**<0.001**	324 (51)	114 (75)	140 (42)	70 (47)	**<0.001**
Highest educational level obtained, n (%) = High[d]	8	1228 (64)	307 (64)	9 (30)	47 (47)	251 (72)	**<0.001**	426 (52)	41 (25)	385 (58)	**<0.001**	495 (79)	144 (97)	214 (65)	137 (91)	**<0.001**
Work with patients or human tissues in the past year, n (%) = Yes[e]	43	605 (32)	171 (36)	19 (68)	32 (32)	120 (35)	**0.001**	321 (39)	96 (62)	225 (34)	**<0.001**	113 (18)	25 (18)	50 (15)	38 (26)	**0.025**
Hospital visits as a patient in the past year, n (%) = Yes	2	172 (9)	74 (15)	2 (7)	18 (18)	54 (16)	0.332	42 (5)	2 (1)	40 (6)	**0.024**	56 (9)	9 (6)	38 (12)	9 (6)	**0.046**
Hospital visits as a professional in the past year, n (%) = Yes	2	59 (3)	31 (6)	0 (0)	6 (6)	25 (7)	0.299	14 (2)	0 (0)	14 (2)	0.131	14 (2)	0 (0)	7 (2)	7 (5)	**0.023**
Use of antibiotics in the past year, n (%) = Yes	4	454 (23)	147 (31)	7 (23)	27 (27)	113 (32)	0.372	147 (18)	17 (11)	130 (20)	**0.010**	160 (25)	31 (21)	83 (25)	46 (30)	0.156
Farm visits in the past year, n (%) = Yes	9	181 (9)	85 (18)	10 (33)	14 (14)	61 (17)	**0.050**	79 (10)	25 (16)	54 (8)	**0.005**	17 (3)	2 (1)	7 (2)	8 (5)	0.067
Travel to high risk areas for AR in the past year, n (%) = Yes[f]	18	658 (34)	241 (51)	13 (43)	42 (42)	186 (54)	0.083	291 (36)	52 (33)	239 (36)	0.501	126 (20)	45 (30)	32 (10)	49 (33)	**<0.001**
Carriage of ESBL-producing *E. coli*, n (%) = Positive	163	236 (13)	26 (7)	0 (0)	5 (6)	21 (8)	0.218	47 (6)	7 (4)	40 (6)	0.532	163 (28)	27 (23)	118 (36)	18 (12)	**<0.001**
Carriage of ESBL-producing KESC bacteria, n (%) = Positive	163	67 (4)	4 (1)	0 (0)	1 (1)	3 (1)	0.845	4 (0)	3 (2)	1 (0)	**0.029**	59 (10)	12 (10)	35 (11)	12 (8)	0.740

Notes:
This table is a reproduction of the descriptive table of characteristics included in Publication 1.
Bold highlighting means that the estimate was statistically significant at the 0.05 level.
[a]No data from nearby residents were collected in the Netherlands.
[b]Distant residents live at least 1000 m away from a WWTP
[c]Nearby residents live within a 300 m radius from a WWTP.
[d]Educational level according to the International Standard Classification of Education (ISCED): Low = ISCED 0-2 (Pre-primary education to Lower secondary education), High = ISCED ≥3 (Upper secondary education to Doctoral or equivalent).
[e]Work with patients or human tissues in the past year: Includes self-reported contact with patients at work and with human tissues (e.g. blood, urine, sputum, feces, vomit, saliva, or primary cell lines).
[f]Travel to high risk areas for AR in the past year: Includes travels to North Africa, Sub-Saharan Africa, Asia, Central and South America, as well as the European countries Italy, Greece, Bulgaria and Slovenia.
ESBL: Extended-Spectrum Beta-Lactamases.
AR: Antibiotic Resistance.

There were two main challenges in this publication, which I addressed in the statistical analyses of these data using diverse methods. First, because of time constraints, we failed to receive many stool samples in Germany, which led to a high number of missing values in the studied outcome. Since it is very unlikely that participants knew their carriage status when participating in the study, I assumed that these missing values were missing at random (MAR). Therefore, I applied multiple imputation with chained equations to simulate missing values for this and other variables of interest. Second, because participation response was low in both Germany and the Netherlands (in comparison to Romania), I decided to apply inverse probability of sampling weights (IPSW) defined as the inverse of the participation response per country and per participation group. In this study, we found that the odds for carriage of ESBL-producing *E. coli* was higher for both WWTP workers and nearby residents in Romania. Full results of this publication can be found within this doctoral dissertation in the published peer-review paper corresponding to Publication 1. The main strength of this publication is that, as far as we know, this was the first study investigating the potential transmission risk of antibiotic-resistant *E.* coli from a local WWTP as a source point to WWTP workers and nearby residents.

2.3 Publication 2: International travel as a risk factor for carriage of extended-spectrum β-lactamase-producing *Escherichia coli* in a large sample of European individuals - The AWARE Study

In this publication (104), we aimed to explore a wide variety of risk factors for carrying ESBL-producing E. coli, as well as to describe and characterize them. Because here we were interested in all potential risk factors, we decided not to exclude participants based on their work in healthcare, at farms or at slaughterhouses. The investigated risk factors included sociodemographic variables, job history, farm and hospital visits, use of medication, health status, diarrhea frequency, previous surgeries, and how often participants travelled to different geographical continents. All potential risk factors were explored within the last 12 months before answering the questionnaire. The total sample size was 1183 participants: 689 in the Netherlands, 333 in Germany and 161 in Romania.

In our sample, the prevalence of ESBL-producing E. coli was 8% for all three countries. The lowest estimate was in the Netherlands (6%) while the highest was in Romania (13%). In Germany, the prevalence of ESBL-producing E. coli was 8%. Logistic regression models showed that international travel to Asia and Africa is a risk factor for carriage of ESBL-producing *E. coli*: The adjusted OR and corresponding 95% CI for Asia was 4.08 (1.97–8.43), for Northern Africa was 4.03 (1.67–9.68), and for Sub-Saharan Africa was 4.60 (1.60–13.26). According to these data, none of the other geographical areas nor of the other considered potential risk factors seemed to be associated with the carriage of ESBL-producing *E. coli*. Full results of this publication can be found within this doctoral dissertation in the published peer-review paper corresponding to Publication 2.

As the common saying goes: “the absence of evidence is not evidence of absence”. Finding negative results for other potential confounders such as antibiotics use does not indicate that these are not potential risk factors, but rather that these risk factors are better explored in specific high-risk populations such as patients, or using very large sample sizes from the general population, i.e. to increase the statistical power in order to identify an effect. Rather, our results mean that the effect of international travel to Asia and Africa is so determinant to the carriage of ESBL-producing *E. coli*, that it is possible to estimate such parameters even in a sample stemming from the general population. We concluded that travelling to Africa and Asia within the last year increases the chances of being a carrier of ESBL-producing E. coli in the gut microbiota.

2.4 Appendix 1: Antibiotic Resistance in Wastewater Treatment Plants and Transmission Risks for Employees and Residents: The Concept of the AWARE Study

This publication (102) describes the design and scope of the AWARE study and details the methods planned to correctly characterize the potential risk of being exposed to AR bacteria such as ESBL-producing *E. coli* at or around WWTPs. I decided to include it as part of this doctoral dissertation because it expands the information about the methodology used for the whole AWARE study, complements the methodology of the two main publications, and shows part of the work that I did in terms of data collection. For more information about my personal contribution to this publication, see 1. Contributions to the Individual Publications.

2.5 Conclusions

With the work presented in this doctoral dissertation, I was able to estimate that the prevalence of ESBL-producing *E. coli* in the gut microbiota of study participants was higher in Romania than in Germany or the Netherlands. I found out that the carriage of ESBL-producing E. coli in the gut microbiota of WWTP workers and people living close to a WWTP in Romania is higher when compared to people living more than 1 km away from a local WWTP. By focusing on the subset of distant residents, which I considered to approximate the general population in the three countries, I was able to show that international travel to Africa and Asia within the past 12 months does increase the chance of carrying ESBL-producing *E. coli* in the gut microbiota. To the best of my knowledge, AWARE is the first study investigating the potential transmission risk of antibiotic-resistant bacteria from WWTPs as point sources to WWTP workers and nearby residents. These results represent a unique contribution to the growing body of scientific evidence in the topic of antibiotic resistance in occupational and environmental epidemiology.

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3. Publication 1

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ORIGINAL ARTICLE

Carriage of ESBL-producing Enterobacterales in wastewater treatment plant workers and surrounding residents — the AWARE Study

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Abstract
To investigate whether wastewater treatment plant (WWTP) workers and residents living in close proximity to a WWTP have elevated carriage rates of ESBL-producing Enterobacterales, as compared to the general population. From 2018 to 2020, we carried out a cross-sectional study in Germany, the Netherlands, and Romania among WWTP workers (N = 344), nearby residents (living ≤ 300 m away from WWTPs; N = 431) and distant residents (living ≥ 1000 m away = reference group; N = 1165). We collected information on potential confounders via questionnaire. Culture of participants' stool samples was performed with ChromID®-ESBL agar plates and species identification with MALDI-TOF–MS. We used logistic regression to estimate the odds ratio (OR) for carrying ESBL-producing *E. coli* (ESBL-EC). Sensitivity analyses included stratification by country and interaction models using country as secondary exposure. Prevalence of ESBL-EC was 11% (workers), 29% (nearby residents), and 7% (distant residents), and higher in Romania (28%) than in Germany (7%) and the Netherlands (6%). Models stratified by country showed that within the Romanian population, WWTP workers are about twice as likely (aOR = 2.34, 95% CI: 1.22–4.50) and nearby residents about three times as likely (aOR = 3.17, 95% CI: 1.80–5.59) to be ESBL-EC carriers, when compared with distant residents. In stratified analyses by country, we found an increased risk for carriage of ESBL-EC in Romanian workers and nearby residents. This effect was higher for nearby residents than for workers, which suggests that, for nearby residents, factors other than the local WWTP could contribute to the increased carriage.

Keywords Antimicrobial resistance · Antibiotic resistance · ESBL-producing *E. coli* · Wastewater treatment plants · Environmental exposure

Introduction

Antibiotic resistance (AR) is currently one of the most important threats to public health and clinical medicine. In some regions, current AR rates are alarmingly high, with 58.4% of *Escherichia coli* (*E. coli*) isolates reported in 2018 to the European Antimicrobial Resistance Surveillance Network being resistant to at least one antibiotic group under surveillance (i.e. aminopenicillins, fluoroquinolones, third-generation cephalosporins, aminoglycosides and carbapenems) [1]. This is partly due to the use, overuse, and misuse of antibiotics by healthcare professionals and patients, but also in animal husbandry and agriculture [2–6]. Antibiotic resistant bacteria (ARB) can be introduced into the environment by different routes [7], including wastewater from the general human population [8–15]. These residual waters arrive and are collected at municipal wastewater treatment plants (WWTPs). Enteric ARB such as *E. coli*, as well as *Klebsiella* spp., *Enterobacter* spp., *Serratia* spp., and *Citrobacter* spp. (KESC) have been found in water [16–22] and air [23–25] samples from WWTPs. Moreover, the WWTPs effluents can discharge ARB into nearby water bodies because eliminating ARB is not part of the current wastewater treatment processes, which focus instead on reducing nutrient loads and pathogens to the receiving

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surface water. While some studies have reported either no changes in relative abundances of ARB [26] or a decrease in absolute and relative abundance of ARGs [27–29], other studies have reported an increased relative prevalence of ARB after wastewater treatment processes, in comparison to the untreated wastewater entering the plant [16, 17, 22, 30–38]. These aspects make WWTPs potential transmission hubs for the spread of ARB into the environment [39].

It has been proposed that ARB could be transmitted to humans by the air or wastewater at the WWTPs through different exposure routes including ingestion of droplets, hand-to-mouth contact, or inhalation of aerosols [21–24]. Further, an increased prevalence of gastrointestinal and respiratory diseases [40], as well as high levels of antibodies against bacteria, viruses, and parasites in WWTP workers, suggests an increased exposure to these pathogens [41–43]. Under this scenario, and extending this idea to AR, WWTP workers would be at a high risk of exposure to ARB. Furthermore, and considering that extended-spectrum betalactamase (ESBL)-producing *E. coli* (ESBL-EC) can be found up to 150 m both up- and downwind away from animal farms [44], nearby residents living in close proximity to WWTPs could also be highly exposed to these ARB. However, to our knowledge, no large-scale study has yet been carried out in humans potentially at risk of carriage of antibiotic resistant Enterobacterales working at or living close to WWTPs. Such studies are critical to aid our current understanding of the exposure status of humans working at or living around WWTPs, and to devise preventive strategies and interventions to reduce this potential exposure.

Therefore, in the present study, we aimed at investigating whether WWTP workers and residents living in close proximity to a WWTP have elevated carriage rates of ESBL-producing Enterobacterales, as compared to the general population. Our hypothesis is that the risk of carrying ESBL-producing Enterobacterales increases with proximity to the WWTP.

Materials and methods

Study design and population

The project "Antibiotic Resistance in Wastewater: Transmission Risks for Employees and Residents around Wastewater Treatment Plants (AWARE)" is a cross-sectional study, with data collection carried out from September 2018 to March 2020 in three European countries with different background prevalences for AR: Germany, the Netherlands, and Romania. A thorough description of the study methodology can be found elsewhere [45]. Briefly, our target population consisted of two exposed groups working at or living in close proximity to wastewater treatment plants (WWTP workers and nearby residents) and one unexposed population of distant residents. Nearby residents were defined as living within a 300-m radius from a WWTP, while distant residents were defined as living more than 1000 m away from a WWTP. Data on nearby residents was only collected in Germany and Romania, while data on WWTP workers and distant residents was collected in all three countries. The process of recruiting participants per country is described as follows.

Germany

We generated a sampling frame of WWTPs and ranked them in descending order based on number of employed workers and of estimated nearby residents in their vicinity to maximize the chances of achieving the minimum sample size for these two exposed groups. Out of 18 eligible WWTPs with the largest number of employed workers and nearby residents, eight were interested in participating and were thus invited into the study. Of these eight plants, six were willing to participate, of which one had too few workers and was thus not eligible, one could not participate anymore because of the situation regarding COVID-19 in early 2020, and one was selected as a pilot phase plant because it had a lower number of workers and nearby residents (Fig. 1). The remaining three plants were enrolled in full participation.

After a pilot phase examining the feasibility of the study methods, a total of 137 workers employed at three WWTPs in Southern Germany were invited to participate in our study (response 22%). For nearby and distant residents of each of these three WWTPs, postal addresses were obtained from the local civil registries whenever possible, and all individuals living at each household were invited to participate in our study via postal service. In study locations where this was not possible, we generated a sampling frame of addresses within the specified distances to the WWTP for nearby and distant residents using Google Maps™, and went door-to-door delivering invitation letters to mailboxes. In addition to the invitation letter, two reminders were sent to non-responders. In parallel, local newspapers published an article about the project on the same week that the participants received the invitation letter. We also carried out a recruitment campaign via Facebook, targeting potential participants within the desired age range and located at the study sites. All participants who successfully completed the study were eligible for a raffle of shopping vouchers with a total value of 1500 EUR. In total, we invited 1453 nearby residents within the eligible age range (response 6.95%) and 3153 distant residents (response 11%).

The Netherlands

In the Netherlands, WWTPs are managed by regional water authorities called waterboards. Our unit of recruitment for

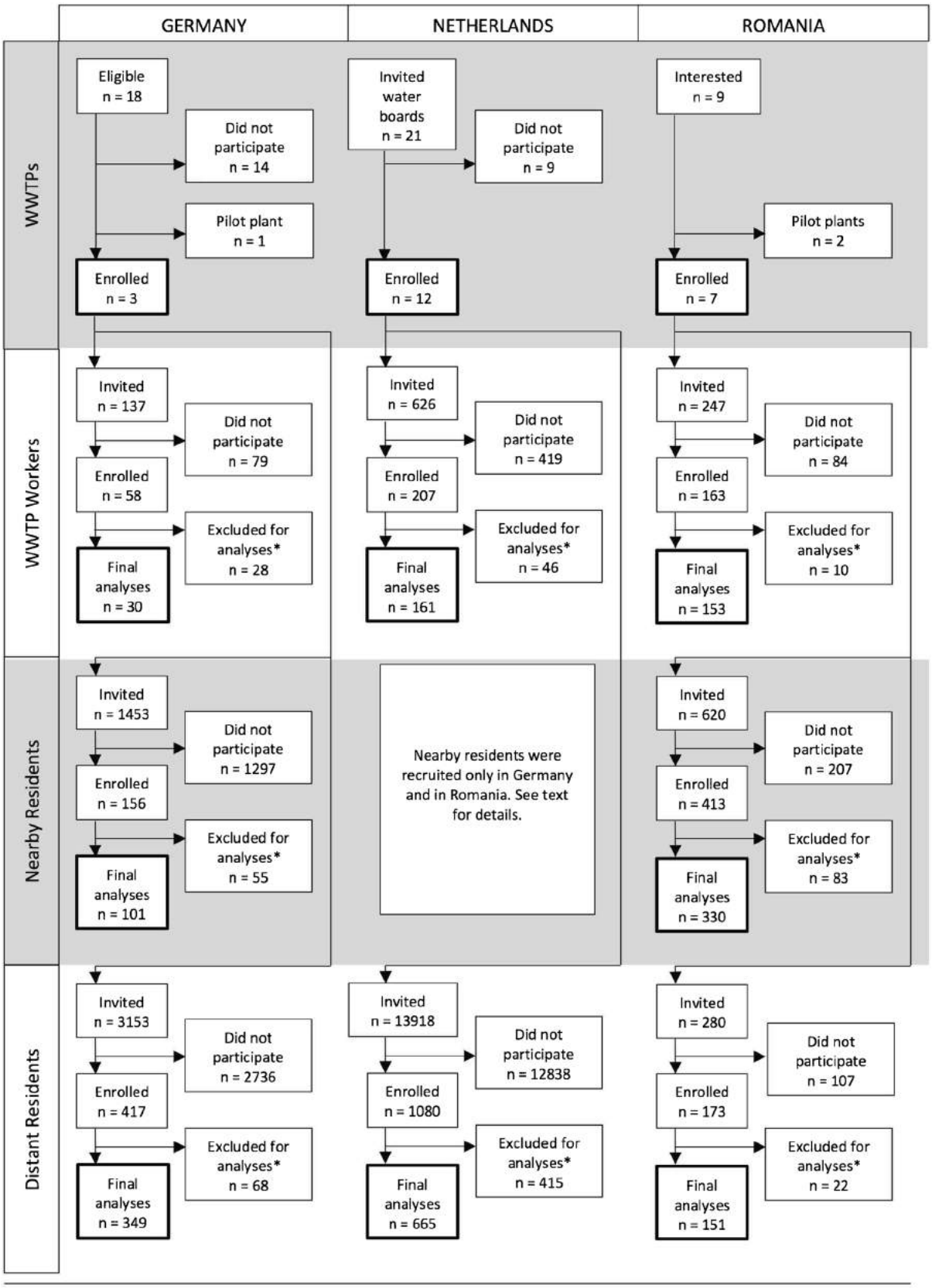

Fig. 1 Flow diagram of the recruitment process, AWARE Study, 2021

the Netherlands was therefore the waterboard and not the WWTP. Of a total of 21 waterboards across the whole country, 12 were interested in participating in the study. Overall, 626 WWTP workers were invited to participate using a combination of WWTP visits for presenting the study plus invitations by e-mail in ten out of these twelve WWTPs, and using only e-mail invitations in the remaining two plants (response 26%). We did not carry out data collection for residents living in close proximity to WWTPs in the Netherlands. For distant residents, general practitioners (GP) practices located 2 to 5 km away from the selected WWTPs were identified and these GPs were invited to cooperate with us as their practices served as a collection and preservation point for stool samples. Using ArcGis [46], we then identified all postal addresses within a 500-m radius from the cooperating GP practices, and then, using the Dutch Personal Records Database, we randomly retrieved the contacting information of potential participants living in 300–500 addresses surrounding each GP practice. A total number of 13,918 individuals living at these addresses received an invitation letter per postal service, of which 1080 responded to the invitation (recruitment response 7.8%). Of these 13,918 invited people, 10,448 individuals were between the age of 16 and 67 years old and thus eligible by age (response among eligible individuals 6.4%). All participants completing the study received a gift card worth 20 EUR.

Romania

WWTP operators were recruited through a formal letter containing information about the project and an invitation to join the study. Nine plants were invited, of which two were pilot plants, and all of them were ultimately enrolled in the study. WWTP workers from participating plants were contacted by their respective operators and invited to participate. A total number of 247 workers were reached (response 62%). Nearby and distant residents were invited to participate using the door-to-door approach. Further, potential participants in public places like streets, parks, and markets in the vicinity of WWTPs were also addressed orally and invited to participate, given that they were eligible. In total, we contacted 620 nearby and 280 distant residents within the eligible age range (response 53% and 54%).

Data collection

Exposure of interest

We consider ingestion of droplets, hand-to-mouth contact, or inhalation of aerosols the main exposure routes for WWTP workers. Nearby residents would be exposed through inhalation of aerosols. Therefore, we used the variable participation group (WWTP worker, nearby resident, distant resident) as a proxy variable for the exposure. We defined WWTP workers as the highest exposed group followed by nearby residents as the second most-exposed group, while distant residents served as an unexposed comparison group. Nearby residents were defined as persons living fewer than 300 m away from the WWTP. Distant residents were defined as persons living further than 1000 m away from any WWTPs.

Outcome of interest

The main outcome of interest was the presence of ESBL-EC in stool samples, reported binarily (positive/negative). A secondary outcome of interest was the presence of bacteria from the *Klebsiella*, *Enterobacter*, *Citrobacter*, and *Serratia* (KESC) group in stool samples, also reported binarily (positive/negative). In Germany and Romania, only participants who successfully filled in the study questionnaire were sent a stool sample kit. In the Netherlands, enrolled participants were required to hand in a stool sample before receiving a link to fill in the online questionnaire. Nearby and distant residents received a stool sample collection kit by postal service, whereas workers received it at their workplace. Each participant was asked to record the date and time of stool sample collection, maintain the sample refrigerated (temperature ranging from 2 to 8 °C), and bring it to the closest collection point (WWTPs or main train station in Germany, WWTPs or GP offices in the Netherlands, home visits in Romania). Samples were transported to the laboratory in cooling boxes within 24 h after sampling, where they were stored at 4 °C, and processed within 24–48 h after sampling.

At the local laboratories in Germany, the Netherlands and Romania, all the stool samples were inoculated directly onto the following culture media: ChromID® ESBL (for ESBL-EC), TBX (in the Netherlands and Romania) or MacConkey (in Germany) (for *E. coli*), and incubated at 36 °C ± 1 °C for 24–48 h. In case of positive results, 2 separate isolates belonging to the ESBL-EC phenotype were collected from the ChromID® ESBL plate, screened for antibiotic resistance and identified by MALDI-TOF MS (Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry). Participants with a negative stool culture on TBX/MacConkey were excluded from further analyses.

Confounding variables

Information on confounding variables was obtained from eligible individuals through an online questionnaire exploring sociodemographic characteristics, work history including contact with animals during farming or slaughterhouse activities, contact with patients or human tissues at work, international travels, use of antibiotics, hospital visits, and

health condition (personal history of surgery, hospitalizations, chronic diseases, antibiotic and antacid intake, diarrhea, respiratory health, and self-reported health status), all in the past 12 months [45].

Educational level was asked using the educational structure of each country and then dichotomized using the International Standard Classification of Education (ISCED) [47–49] into low (0–2 ISCED points, i.e. pre-primary education to lower secondary education) and high (more than 2 ISCED points, i.e. upper secondary education to Doctoral or equivalent).

Work with patients or human tissues was constructed by merging the information of two separate survey questions: "In your current job, how often have you typically had direct interaction or contact with patients within in the last 12 months?" and "How often have you worked with human tissue, blood, body fluids (urine, feces, vomit, sputum, saliva) or primary cell lines within the last 12 months?" Each question could be answered with a frequency scale (never, rarely, sometimes, often, always). If the participant had answered rarely, sometimes, often or always in either of the two questions, a "yes" was assigned. Else, a "no" was assigned. Use of antibiotics was assessed with the question "Have you taken an antibiotic within the last 12 months?" to which possible answers were "Yes," "No," and "Do not know." Participants answering "Do not know" were assigned into the "No" category.

When asked about international travel, participants were asked to provide information about the region where they had been in the past year: Europe, Asia, North Africa, Sub-Saharan Africa, North America, Central America and Mexico, South America, and Australia and Oceania. For each of these regions, participants could state the frequency of travel within the last year: never, once, 2 to 3 times, more than 3 times, I don't know. Additionally, if the participant reported travels to Europe, they were asked about travels to specific European countries with a high background prevalence of ESBL-EC: Italy, Slovenia, Bulgaria, and Greece (yes/no). Travels to high-risk areas for ESBL was defined as reporting travels to at least one of the following areas or countries within the past year: Asia, North Africa, Sub-Saharan Africa, Central America and Mexico, South America, Italy, Slovenia, Bulgaria, and Greece.

Statistical analyses

To present summary statistics for the descriptive characteristics of the study population, numerical variables (i.e. age) were assessed visually for normality using histograms and are presented as mean ± standard deviation if normally distributed or as median ± inter-quartile range if non-normally distributed. Categorical variables are presented using absolute and relative frequencies. Either chi-square of Fisher's exact test was used for bivariate hypothesis testing of categorical variables, depending on cell counts.

We assume that the missing values in the outcome of interest are missing at random because it is highly unlikely that participants would know their personal status of ESBL-EC in stools beforehand. We therefore proceeded to simulate missing values for this outcome and other variables of interest where the missingness mechanism was at random or completely at random by using multiple imputation with chained equations [50]. With twenty iterations per dataset, we generated a total of ten imputed datasets, from which we estimated regression models whose estimates were then pooled and reported. Because of the differences in participation response across countries, we weighted our study population using inverse probability of sampling weights [51]. Weights were defined as the inverse of the participation response per country and per participation group.

The direct causal effect of participation group (WWTP worker, nearby resident, distant resident) as a proxy for exposure routes (ingestion of droplets, hand-to-mouth contact, or inhalation of aerosols) in and around the local WWTP on the presence of ESBL-EC in participants' stool samples (no/yes) was estimated using logistic regression models. We present unweighted crude and adjusted estimates, weighted crude and adjusted estimates, and their corresponding 95% confidence intervals in graphical form. Sensitivity analyses included models stratified by country, an interaction model with country as a secondary exposure, and models stratified by participation group.

Variable selection for the models was done using a combination of experts' opinion from within the AWARE consortium, evidence in the current literature, and the use of Directed Acyclic Graphs (DAGs) [52, 53] (Fig. 2). All analyses were done in R version 3.5.0 and up [54] using the following R packages: epiR [55], mice [56], mitml [57], mitools [58], and survey [59, 60].

Results

Descriptive characteristics of the study population

A total of 1940 participants across the three countries were eligible for analyses, with 25% of participants from Germany (n = 480), 43% from the Netherlands (n = 826), and 33% from Romania (n = 634, Table 1). The majority of the population was middle-aged (median age 49 years, IQR 36–58), female (52%), and highly educated (64%). Across the three countries, WWTP workers were mostly men and the majority reported contact with human tissues, which we attribute to the presence of human feces in wastewater.

In Germany, approximately two-thirds of the WWTP workers reported working with human tissues (68%) in

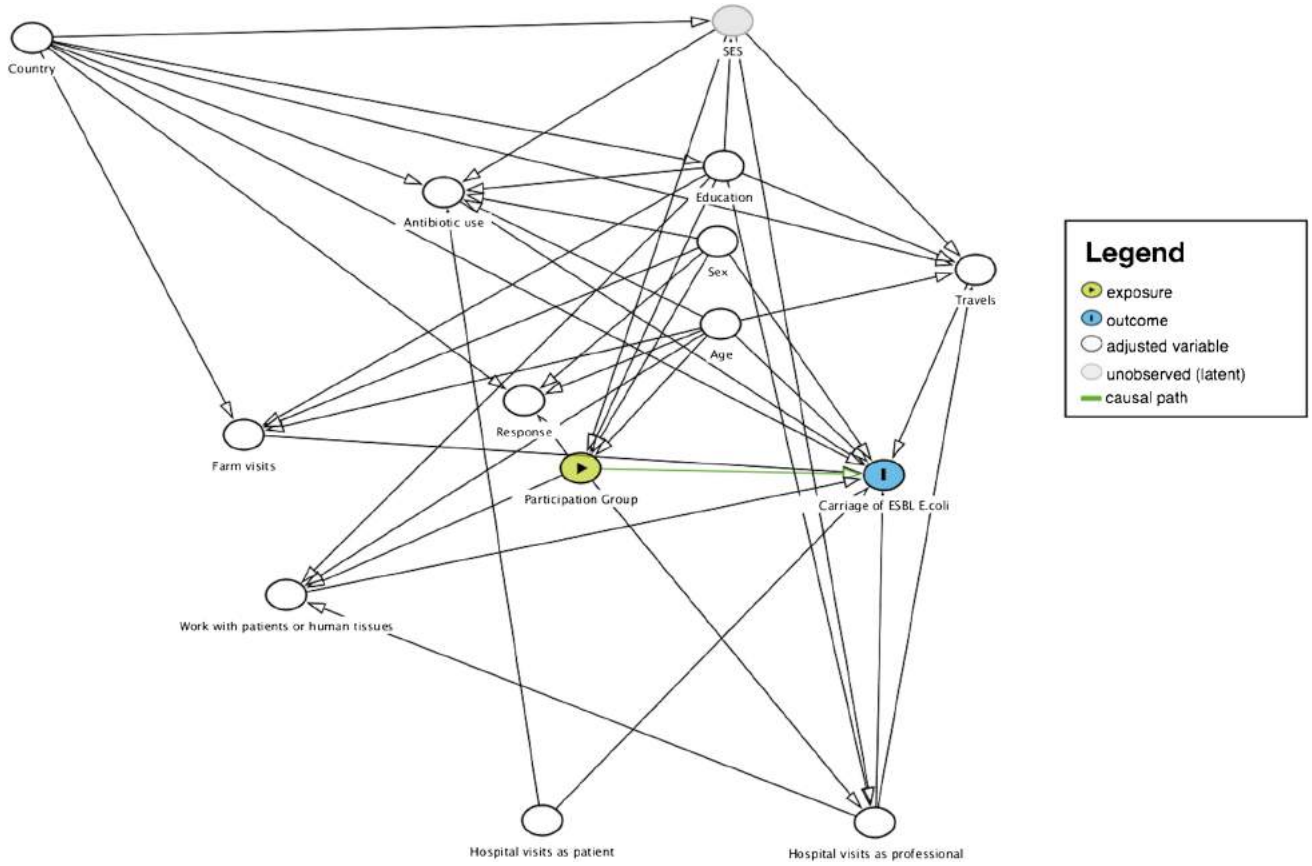

Fig. 2 Directed Acyclic Graph (DAG) for the direct effect of participation group (wastewater treatment plant–WWTP–worker, nearby resident, distant resident) as a proxy for exposure routes (ingestion of droplets, hand-to-mouth contact, or inhalation of aerosols) in and around the local WWTP on the presence of ESBL-producing *E. coli* in stool samples, AWARE Study, 2021

contrast to nearby and distant residents, where approximately a third of each group reported this type of contact at work (32% and 35%, p 0.0015). Distant residents from Germany were more highly educated than nearby residents, and these in turn more than WWTP workers (72%, 47%, and 30%, $p < 0.001$).

In the Netherlands, fewer WWTP workers reported using antibiotics in the past year in comparison to the distant residents (11% vs. 20%, $p = 0.01$) and visiting hospitals as a patient (1.2% vs. 6.0%, $p = 0.02$). More WWTP workers reported visiting farms than distant residents (16% vs. 8.2%, $p = 0.005$).

In Romania, workers were, on average, older (median age among workers 49 [41, 53] vs. median age among distant residents 40 [33, 50] in distant residents) and better educated (97% vs. 91) than distant residents. Also, in comparison to distant residents, nearby residents had a lower level of education (65% vs. 91%) and traveled less to high risk areas for AR (10% vs. 33%).

Carrier status for ESBL-producing Enterobacterales

The overall prevalence of ESBL-EC across the three countries was 13%, with the highest prevalence observed in the Romanian population (28%). The prevalence of ESBL-producing bacteria of the KESC group across countries was 3.8%, with the highest value observed also in Romania (10%).

In Germany, ESBL-EC were not detected in stools of any of the workers ($n = 30$), but among 8.4% of distant residents and 5.7% of nearby residents. In the Netherlands, carriage of ESBL-EC was similar in WWTP workers (4.4%) and distant residents (6.0%) ($p = 0.53$). In Romania, the prevalence of ESBL-EC was 23% among workers, 36% among nearby residents, and 12% among distant residents ($p < 0.001$).

Because the prevalence for KESC bacteria was relatively low and thus limiting the statistical power of our inferential analyses, we decided to focus only on the primary outcome: ESBL-EC. The effect of participation

Table 1 Descriptive characteristics of the studied population by country and participation group, n = 1940, AWARE Study, 2021

			Germany					The Netherlands[a]				Romania				
Variable	Missings	Overall	Overall	WWTP worker	Nearby resident[c]	Distant resident[b]	p	Overall	WWTP worker	Distant resident[b]	p	Overall	WWTP worker	Nearby resident[c]	Distant resident[b]	p
n		1940	480	30	101	349		826	161	665		634	153	330	151	
Age, years (median [IQR])	0	49 [36, 58]	47 [35, 57]	52 [44, 55]	48 [35, 58]	45 [34, 56]	0.161	54 [40, 61]	54 [45, 59]	55 [39, 61]	0.710	43 [34, 53]	49 [41, 53]	41 [32, 54]	40 [33, 50]	**< 0.001**
Sex, n (%) = Male	4	938 (48)	211 (44)	24 (80)	51 (50)	136 (39)	**< 0.001**	403 (49)	150 (93)	253 (38)	**< 0.001**	324 (51)	114 (75)	140 (42)	70 (47)	**< 0.001**
Highest educational level obtained, n (%) = High[d]	8	1228 (64)	307 (64)	9 (30)	47 (47)	251 (72)	**< 0.001**	426 (52)	41 (25)	385 (58)	**< 0.001**	495 (79)	144 (97)	214 (65)	137 (91)	**< 0.001**
Work with patients or human tissues in the past year, n (%) = Yes[e]	43	605 (32)	171 (36)	19 (68)	32 (32)	120 (35)	**0.001**	321 (39)	96 (62)	225 (34)	**< 0.001**	113 (18)	25 (18)	50 (15)	38 (26)	**0.025**
Hospital visits as a patient in the past year, n (%) = Yes	2	172 (9)	74 (15)	2 (7)	18 (18)	54 (16)	0.332	42 (5)	2 (1)	40 (6)	**0.024**	56 (9)	9 (6)	38 (12)	9 (6)	**0.046**
Hospital visits as a professional in the past year, n (%) = Yes	2	59 (3)	31 (6)	0 (0)	6 (6)	25 (7)	0.299	14 (2)	0 (0)	14 (2)	0.131	14 (2)	0 (0)	7 (2)	7 (5)	**0.023**
Use of antibiotics in the past year, n (%) = Yes	4	454 (23)	147 (31)	7 (23)	27 (27)	113 (32)	0.372	147 (18)	17 (11)	130 (20)	**0.010**	160 (25)	31 (21)	83 (25)	46 (30)	0.156
Farm visits in the past year, n (%) = Yes	9	181 (9)	85 (18)	10 (33)	14 (14)	61 (17)	**0.050**	79 (10)	25 (16)	54 (8)	**0.005**	17 (3)	2 (1)	7 (2)	8 (5)	0.067

Table 1 (continued)

Variable	Missings	Overall	Germany					The Netherlands[a]				Romania				
			Overall	WWTP worker	Nearby resident[c]	Distant resident[b]	p	Overall	WWTP worker	Distant resident[b]	p	Overall	WWTP worker	Nearby resident[c]	Distant resident[b]	p
Travel to high risk areas for AR in the past year, n (%) = Yes[f]	18	658 (34)	241 (51)	13 (43)	42 (42)	186 (54)	0.083	291 (36)	52 (33)	239 (36)	0.501	126 (20)	45 (30)	32 (10)	49 (33)	**< 0.001**
Carriage of ESBL-producing E. coli, n (%) = Positive	163	236 (13)	26 (7)	0 (0)	5 (6)	21 (8)	0.218	47 (6)	7 (4)	40 (6)	0.532	163 (28)	27 (23)	118 (36)	18 (12)	**< 0.001**
Carriage of ESBL-producing KESC bacteria, n (%) = Positive	163	67 (4)	4 (1)	0 (0)	1 (1)	3 (1)	0.845	4 (0)	3 (2)	1 (0)	**0.029**	59 (10)	12 (10)	35 (11)	12 (8)	0.740

Bold data indicates a *p*-value equal to or under 0.05. *ESBL* Extended-Spectrum Beta-Lactamases, *AR* Antibiotic Resistance

[a]No data from nearby residents were collected inthe Netherlands

[b]Distant residents live at least 1000 m away from a WWTP

[c]Nearby residents live within a 300 m radius from a WWTP

[d]Educational level according to the International Standard Classification of Education (ISCED): Low = ISCED 0–2 (Pre-primary education to Lower secondary education), High = ISCED ≥ 3 (Upper secondary education to Doctoral or equivalent)

[e]Work with patients or human tissues in the past year: Includes self-reported contact with patients at work and with human tissues (e.g. blood, urine, sputum, feces, vomit, saliva, or primary cell lines)

[f]Travel to high risk areas for AR in the past year: Includes travels to North Africa, Sub-Saharan Africa, Asia, Central and South America, as well as the European countries Italy, Greece, Bulgaria and Slovenia

group (WWTP worker, nearby or distant resident) on the carriage of ESBL-EC varied by country (Online Resource Table 1). Overall, the proportion of WWTP workers and nearby residents with a positive stool sample for ESBL-EC was higher than that of distant residents (11% and 29% vs. 7.5%, $p < 0.001$). This result was driven by the Romanian population (23% and 36% vs. 12%, $p < 0.001$), while there were no statistically significant differences between participation groups in the proportions of positive ESBL-EC carriers either in Germany (0.0% and 5.7% vs. 8.4%, $p = 0.22$) or in the Netherlands (4.4% vs. 6.0%, $p = 0.53$).

Statistical models

Across the three countries, the unweighted crude odds ratio for the carriage of ESBL-EC among WWTP workers was 1.71 (95% CI: 1.12–2.61). Among nearby residents, it was 4.95 (95% confidence interval, CI: 3.63–6.73), compared to the unexposed group (Fig. 3). These unweighted estimates changed to 1.17 (95% CI: 0.74–1.86) for WWTP workers and 2.24 (95% CI: 1.50–3.37) for nearby residents upon adjustment for age, sex, education, country, travels to high risk areas for AR, working with human tissues, antibiotic use, farm visits, hospital visits as patients, and hospital visits as a professional. After applying inverse probability of sampling weights for the response in each country and in each participation group, crude estimates changed to 1.28 (95% CI: 0.82–2.00) among workers and to 2.46 (95% CI: 1.65–3.69) among nearby residents, while the adjusted estimates changed to 0.76 (95% CI: 0.44–1.29) and 1.47 (95% CI: 0.83–2.59), respectively.

Although we could not estimate an effect of exposure within the German and the Dutch subpopulations (Table 2), models stratified by country showed that, within the Romanian population, WWTP workers were about twice as likely (adjusted OR, aOR = 2.34, 95% CI: 1.22–4.50) and nearby residents about three times as likely (aOR = 3.17, 95% CI: 1.80–5.59) to be ESBL-EC carriers, when compared with distant residents.

Additionally, and according to our weighted and adjusted model, participants who reported traveling to high risk areas for AR in the past 12 months were almost twice as likely to have a positive result for ESBL-EC in stool samples, as compared to participants who did not travel to these high-risk areas (aOR 2.06, 95% CI: 1.33–3.19). None of the other covariates showed a statistically significant effect (see Online Resource Table 2 and Online Resource Fig. 1). The magnitude and direction of these estimates, as well as their confidence intervals, were fairly conserved in the stratified models by participation group (see Online Resource Fig. 2).

Missing values

The highest proportion of missing values was found in the carriage of ESBL-EC (n = 163, 8.4%), driven mostly by the German population (n = 114, 24%, Table 1). A comparison of crude and adjusted odds ratios (OR) along with 95% CI for logistic regression models with complete case analysis and with the imputed dataset showed that the direction of effect did not change after imputation (Online Resource Table 3).

Discussion

Across the three countries, we found no evidence of an increased risk for carriage of ESBL-EC neither in WWTP workers nor in residents living in close proximity to these WWTPs, as compared to the general population. We did find, however, evidence of increased odds for carriage of ESBL-EC in WWTP workers and in nearby residents in the Romanian population. Contrary to what we initially hypothesized, the effect for nearby residents was higher than the effect for WWTP workers in Romania.

An increased background prevalence of ESBL-EC in Romania, supported by our data, could be a risk factor for ESBL-EC carriage that sets the Romanian study population apart from the German and the Dutch. Additionally, travel to high-risk areas for AR has been identified as a risk factor for the carriage of ESBL-producing Enterobacterales because of the increased background prevalence of AR in some travel destinations [61–65]. Our data show that participants travel differently to high-risk areas for AR depending on their original country of residence. In Germany, our data collection took place in the south of the country where residents tend to choose Italy or Slovenia for their vacations because of the close geographical proximity, resulting in approximately half of the German participants reporting travels to high-risk areas for AR (Table 1).

Finding a higher ESBL-EC estimate for nearby residents than for WWTP workers in Romania, even after adjustment for other potential confounders and sources of exposure, suggests that the main source of exposure for nearby residents might not be the local WWTP. Potential sources of exposure for which we did not collect data and that might uniquely affect nearby residents in Romania but not WWTP workers are mentioned as follows. Risk factors for acquiring community-associated ESBL infection include use of corticosteroids [66] and personal history of diabetes mellitus [66, 67], which is relevant for our study because, at 11.6%, Romania is one of the countries with the highest prevalence of diabetes mellitus in Europe [68]. Person-to-person transmission of ESBL-producing Enterobacterales within households has been documented in Spain [69], the

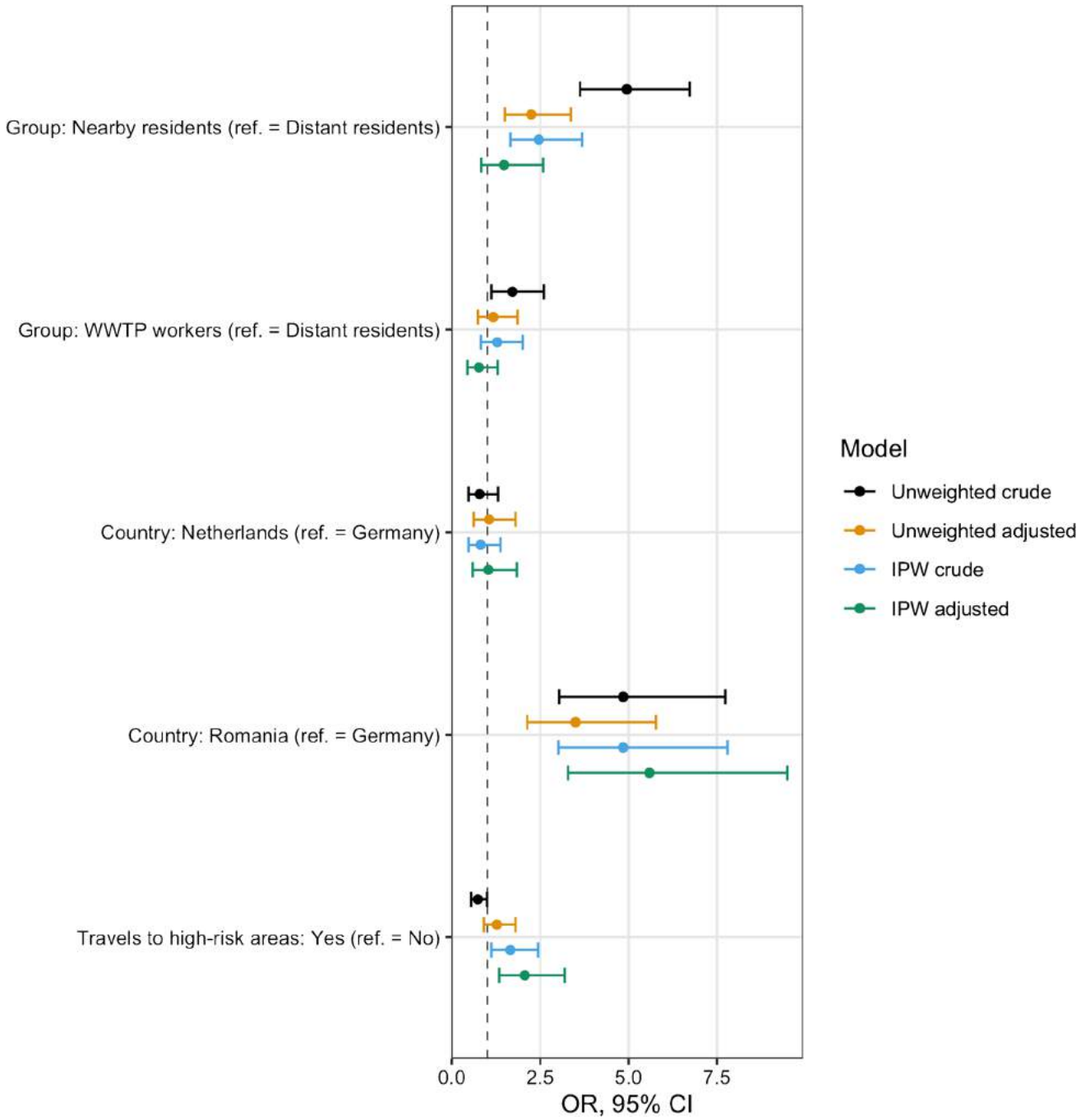

Fig. 3 Comparison of models estimating the effect of participation group (wastewater treatment plant–WWTP–worker, nearby resident, distant resident) as a proxy for exposure routes (ingestion of droplets, hand-to-mouth contact, or inhalation of aerosols) in and around the local WWTP on the presence of ESBL-producing *E. coli* in stool samples, AWARE Study, 2021. Models adjusted for age, sex, education, country, travels to high risk areas, working with human tissues, antibiotic use, farm visits, hospital visits as patient and hospital visits as a professional. IPW: Inverse Probability Weighted model. ref. = Reference level. Travel to high risk areas for AR in the past year includes travels to North Africa, Sub-Saharan Africa, Asia, Central and South America, as well as the European countries Italy, Greece, Bulgaria, and Slovenia. Crude: Model with only the given variable, ignoring potential covariates. Adjusted: Model with the given variable, including all potential covariates in the exposure-outcome relation. Unweighted: Model without applying inverse probability weights (IPW). Weighted: Model applying inverse probability weights (IPW). See text for details

Table 2 Unweighted models for the carriage of ESBL-producing *E. coli*, stratified by country, n = 1940, AWARE Study, 2021

	Germany, n = 482		The Netherlands, n = 828		Romania, n = 608	
	cOR (95% CI) [a]	aOR (95% CI) [b]	cOR (95% CI) [a]	aOR (95% CI) [b]	cOR (95% CI) [a]	aOR (95% CI) [b]
Group: Nearby resident [c]	0.72 (0.27–1.90)	0.81 (0.29–2.30)	[d]	[d]	3.73 (2.18–6.38)	3.17 (1.80–5.59)
Group: WWTP worker	0.00 (0-Inf)[e]	0.00 (0-Inf)[e]	0.71 (0.31–1.62)	0.95 (0.37–2.44)	2.01 (1.08–3.74)	2.34 (1.22–4.50)
Educational level: High[f]	1.72 (0.71–4.17)	1.16 (0.45–2.99)	2.07 (1.10–3.89)	1.85 (0.95–3.59)	0.46 (0.30–0.70)	0.66 (0.41–1.04)
Sex: Male	0.92 (0.42–1.98)	1.01 (0.45–2.24)	0.92 (0.51–1.65)	0.93 (0.48–1.8)	0.95 (0.67–1.36)	1.05 (0.70–1.56)
Age	0.97 (0.95–1.01)	0.98 (0.95–1.01)	1.01 (0.98–1.03)	1.01 (0.99–1.03)	0.98 (0.96–0.99)	0.98 (0.96–0.99)
Travels to high-risk areas: Yes[g]	2.41 (0.99–5.90)	2.29 (0.90–5.78)	2.03 (1.11–3.69)	1.92 (1.04–3.52)	0.54 (0.32–0.92)	0.75 (0.43–1.32)
Work with patients or human tissues: Yes[h]	0.88 (0.38–2.06)	0.99 (0.40–2.43)	0.70 (0.37–1.32)	0.72 (0.37–1.4)	0.54 (0.32–0.93)	0.59 (0.32–1.07)
Hospital visits as a patient: Yes	0.97 (0.34–2.79)	1.00 (0.33–2.99)	0.39 (0.05–2.91)	0.42 (0.05–3.31)	1.18 (0.65–2.16)	1.02 (0.52–2.03)
Hospital visits as a professional: Yes	0.48 (0.06–3.63)	0.44 (0.05–3.50)	1.28 (0.16–10.04)	1.31 (0.15–11.23)	0.59 (0.13–2.62)	1.20 (0.22–6.46)
Use of antibiotics: Yes	1.19 (0.52–2.72)	1.09 (0.46–2.55)	0.80 (0.35–1.82)	0.86 (0.36–2.02)	0.98 (0.64–1.49)	1.28 (0.77–2.12)
Farm visits: Yes	0.86 (0.32–2.34)	0.99 (0.35–2.83)	1.13 (0.43–2.95)	1.34 (0.5–3.56)	0.00 (0-Inf)[j]	0.00 (0-Inf)[j]

ESBL Extended-Spectrum Beta-Lactamases, *AR* Antibiotic Resistance

[a]cOR: crude odds ratio

[b]aOR: adjusted odds ratio

[c]Nearby residents live within a 300 m radius from a WWTP

[d]Data on Nearby residents in the Netherlands was not collected

[e]Not possible to estimate the OR for WWTP workers because all workers in Germany had a negative stool sample result for ESBL-producing *E. coli*

[f]Educational level according to the International Standard Classification of Education (ISCED): Low = ISCED 0–2 (Pre-primary education to Lower secondary education), High = ISCED ≥ 3 (Upper secondary education to Doctoral or equivalent)

[g]Travel to high risk areas for AR in the past year: Includes travels to North Africa, Sub-Saharan Africa, Asia, Central and South America, as well as the European countries Italy, Greece, Bulgaria and Slovenia

[h]Work with patients or human tissues in the past year: Includes self-reported contact with patients at work and with human tissues (e.g. blood, urine, sputum, feces, vomit, saliva, or primary cell lines)

[j]Not possible to estimate the OR for farm visits because all participants who stated visiting a farm in the past year had a negative stool sample result for ESBL-producing *E. coli*

Netherlands [70], and the USA [71], even showing identical strains between patients who had community-acquired infections with ESBL-producing Enterobacterales and their household members [72]. Additionally, ethnicity encodes cultural, social, and health behaviors that could result in a higher carriage rate for ESBL-EC [73]. From the door-to-door visits, differences in household size, sociodemographic characteristics, and underlying comorbidities were observed for nearby residents in Romania, although not systematically recorded. Therefore, these risk factors might differ between exposure groups in Romania at a greater degree than in the other countries.

Within the Romanian population, there is also a striking difference in travels to high-risk areas for AR depending on their participation group: although the proportion of participants among WWTP workers and the distant residents is similar regarding travels to high-risk areas for AR (30% and 33%), the proportion of nearby residents traveling to these high-risk areas for AR was, in comparison, low (10%). We observed a similar trend regarding educational level, where the proportion of highly educated participants in Romania was higher for WWTP workers and distant residents (97% and 91%) than for nearby residents (65%). In fact, when considering country of residence as an interaction term

for the effect of participation group on carriage of ESBL-EC (Online Resource Table 4), the effect of Romania as country of residence alone disappeared (aOR 1.55, 95% CI: 0.79–3.05), while the effect of being a nearby resident in Romania carried the observed effect (aOR 5.49, 95% CI: 1.79–16.80). As frequency of travels and educational levels are proxies for socio-economic status (SES), we suspect that nearby residents in Romania have a lower SES, which would then affect our exposure-outcome relation. Although we did not directly collect data about SES, the constructed DAG (Fig. 2) confirmed that adjusting for other potential confounders is enough to find an unbiased estimate for the direct causal effect of proximity to WWTP (defined by participation group) on carriage of ESBL-EC. In our study, we did not measure the full extent of SES (only partially by e.g. education). Thus, SES is an unobserved confounder of the causal effect of participation group on carriage of ESBL-EC. It was therefore not possible to calculate an unbiased total effect of the exposure-outcome relation. However, adjusting for age, sex, education, country, travels to high risk areas for AR, antibiotics use, farm visits, work with patients or tissues, hospital visits as patients, and hospital visits as a professional made it possible to estimate the direct causal effect.

Strengths and limitations

As far as we know, and despite the abundance of studies analyzing ARB in water and air samples from WWTPs [21–24], this is the first study investigating the carriage of ESBL-producing Enterobacterales in humans hypothesized to be exposed through ingestion of droplets, hand-to-mouth contact, or inhalation of aerosols due to close proximity to a WWTP, either from working at a WWTP or from living in the surroundings. Several characteristics make the AWARE Study unique in its design. Data collection was conducted in three European countries with different background prevalences for AR. We explored the exposure-outcome relation defining two exposed groups and one comparison group, we followed a systematic sampling of participants adapted to the local regulations and logistical capabilities, we used reminders and incentives to increase participation, we developed our study questionnaire within a multidisciplinary team of experts, we used validated questions whenever possible, we conducted a pilot study to assess the feasibility of our methods, we conducted quality control processes for data input and data cleaning processes, we used standardized operating procedures (SOPs) in all three locations to guarantee laboratory methods to be comparable, and used positive controls for culture analyses. Additionally, we avoided using data-driven methods for variable selection. Instead, we conducted a thoughtful identification of potential confounders a priori with the help of a directed acyclic graph, and we used methods such as multiple imputation and inverse probability of sampling weights to analytically reduce the impact of missing values and low response. Our results are consistent in sensitivity analyses using alternative analytical methods to model our exposure-outcome relation: Traditional unweighted logistic regression models with complete case analysis and imputed analysis (Online Resource Table 3), unweighted stratified models by country (Table 2), model using country of residence as an interaction term (Online Resource Table 4).

Our study is, however, not exempt of limitations. Threats to internal validity include the risk of selection bias evidenced by the low participation response, especially in Germany and the Netherlands, for which we decided to use inverse probability of sampling weights. In our study, we suspect that the reasons for the observed low response in WWTP workers, nearby, and distant residents from Germany (response 22%, 6.95%, and 11%) and in the Netherlands (response 26%, and 6.4%) when compared with the response in Romania (response 62%, 53%, and 54%), reflect our recruitment methods and possibly background potential cultural differences among the countries. In Germany and in the Netherlands, we invited potential participants using invitation letters sent by postal service, whereas in Romania, we used a door-to-door approach because, in our experience, this method is more effective in Romania than postal letters. Also, studies involving stool samples have been reported to have a low response because of inherent reasons related to the nature of the stool sample [74, 75]. These reasons put our study at risk of selection bias. Inverse probability of sampling weights has been described as an analytical method to adjust for selection bias where weights are assigned based on the factors that generate selection, which in our case is the response, and thus serve to reduce the differences between the study population and the target population [51, 76].

Additionally, after recruitment and applying exclusion criteria for the analysis, we failed to reach the desired sample size for nearby residents in Germany and in Romania. We also failed to reach the desired sample size for workers in Germany at the recruitment stage. This has implications for the statistical power of our study to detect a desired effect, if there is in fact one. A post hoc power test restricted to study participants who completed all study phases (including providing a stool sample) shows that our data provides us with 63% and 75% statistical power to detect a minimum OR of 1.7 in workers and in nearby residents, when compared with distant residents.

Further, our data showed a proportion of 8% of missing values on the ESBL-EC carriage across countries (n = 163). Some of these missing values came from samples collected in the Netherlands (n = 4) and in Romania (n = 45) but the majority of the missing values for stool samples came from Germany (n = 114). Our data collection methods in Germany

shed some light into this large number of missing values: only participants who had already completed the baseline questionnaire received a stool sample kit, and then were given a short time frame to hand in stool samples in person at the previously arranged time and place. These constraints were caused by the limited availability of the local microbiological laboratory to process samples, by the fact that we could not guarantee adequate preservation of samples if sent to the laboratory by postal service, and thus having to collect stool samples in person. Consequently, these values are missing completely at random or, worst case scenario, missing at random conditional on the country of residence. We are confident that randomness is key in the missing mechanism because participants would not have been able to self-assess their AR carriage status a priori. Besides fulfilling the randomness assumption for applying multiple imputation in our data, we performed post hoc imputation diagnostics by comparing models with complete cases vs. after imputation and did not find major differences in the directionality of estimates (Online Resource Table 3).

Finally, we have not included information about the heterogeneity of treatment processes in WWTPs across the three countries, nor have we included specific working conditions at the WWTP for the workers. Actual contact with raw wastewater can be limited to occasional sampling but could pose a higher threat of exposure depending on the time spent at certain locations within the WWTP, the type of activity performed, and the frequency of given activity, which are relevant factors for exposure intensity. Upcoming analyses from our project will include a formal exposure assessment for these study populations based on spatial techniques including physical distance of participants to the WWTPs, working conditions and preventive behavior at work for WWTP workers, and the specific operative characteristics of enrolled WWTPs.

Conclusions

To the best of our knowledge, this is the first study investigating the carriage of ESBL-producing Enterobacterales in humans exposed to antibiotic resistant factors due to close proximity to a WWTP, either from working at a WWTP or from living in the surroundings. Using data collected in Germany, the Netherlands, and Romania, we did not find evidence of an increased risk of carriage of ESBL-producing *E. coli* in WWTP workers or in nearby residents across the three countries, as compared to the general population. We did find an increased risk for carriage of ESBL-EC in the subset of the Romanian population, both in WWTP workers and in nearby residents, which could be at least partially attributed to the local WWTP. However, this effect was higher for nearby residents than for workers, which suggests that, for nearby residents, unmeasured confounding factors could contribute to the increased carriage. Upcoming analyses from this project will perform exposure assessment using spatial techniques, including working conditions at WWTPs and working behavior from WWTP workers, and considering the heterogeneity of WWTP characteristics in terms of treatment efficacy and its consequences for the environment.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10096-021-04387-z.

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Author contribution Study conception and design: MCC, DGJL, KR, DN, AW, AMRH, HS. Fieldwork and data collection: DRM, HB, MB, MK, LM, GP, LW. Microbiology: HB, MB, MK, LM, GP, MCC, BS, AW, AMRH, HS. Data cleaning and analysis: DRM, HB, MB. Interpretation of the data: DRM, FB, HB, CFF, TW, LW, DGJL, KR, AMRH, HS. Drafting of the manuscript: DRM. All authors read and approved the final manuscript.

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Availability of data and material Data and materials are available upon request.

Code availability Code is available upon request.

Declarations

Ethics approval and consent to participate This study was approved by the Ethics Committee of the University of Munich (LMU) (Project-No. 17–734) and by the Research Ethics Committee of the University of Bucharest (Registration-No. 164/05.12.2017). In the Netherlands, this research is exempted for ethical approval under the Dutch Medical Research Involving Human Subjects Act (WMO; Committee: Medisch Ethische Toetsingscommissie, number of confirmation: 19–001/C). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committees and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards, as well as with Directive 95/46/EC, and the 1977 Oviedo Convention of the Council of Europe on human rights and biomedicine. Written informed consent

was obtained from all individual participants included in the study and their legal guardians when applicable.

Conflict of interest The authors declare no competing interests.

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4. Publication 2

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International Travel as a Risk Factor for Carriage of Extended-Spectrum β-Lactamase-Producing *Escherichia coli* in a Large Sample of European Individuals—The AWARE Study

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Abstract: Antibiotic resistance (AR) is currently a major threat to global health, calling for a One Health approach to be properly understood, monitored, tackled, and managed. Potential risk factors for AR are often studied in specific high-risk populations, but are still poorly understood in the general population. Our aim was to explore, describe, and characterize potential risk factors for carriage of Extended-Spectrum Beta-Lactamase-resistant *Escherichia coli* (ESBL-EC) in a large sample of European individuals aged between 16 and 67 years recruited from the general population in Southern Germany, the Netherlands, and Romania. Questionnaire and stool sample collection for this cross-sectional study took place from September 2018 to March 2020. Selected cultures of participants' stool samples were analyzed for detection of ESBL-EC. A total of 1183 participants were included in the analyses: 333 from Germany, 689 from the Netherlands, and 161 from Romania. Travels to Northern Africa (adjusted Odds Ratio, aOR 4.03, 95% Confidence Interval, CI 1.67–9.68), Sub-Saharan Africa (aOR 4.60, 95% CI 1.60–13.26), and Asia (aOR 4.08, 95% CI 1.97–8.43) were identified as independent risk factors

for carriage of ESBL-EC. Therefore, travel to these regions should continue to be routinely asked about by clinical practitioners as possible risk factors when considering antibiotic therapy.

Keywords: antibiotic resistance; antimicrobial resistance; risk factors; ESBL *E. coli*; travels

1. Introduction

Extended-spectrum β-lactamases (ESBLs) are plasmid-mediated enzymes that inactivate β-lactam antibiotics, posing a significant therapeutic challenge in the treatment of both hospital and community-acquired infections [1]. Infections with ESBL-producing *E. coli* (ESBL-EC) often require therapy with last-resort antibiotics, increasing both the risk of resistance and the associated healthcare costs [2,3]. Resistance to last resort antibiotics further limits treatment options and is associated with prolonged hospital stays and increased mortality [4]. An increase in the prevalence of ESBL-EC, in both community and healthcare settings, is now observed worldwide: the current global prevalence of healthy individuals with ESBL-EC from 2003 to 2018 is estimated to be 16.5%; having increased from 2.6% in 2003–2005 to 21.1% in 2015–2018 [5]. In 2019, we estimated the prevalence of these bacteria in the general population of three European countries, and we found it to be 13% in Romania, 8% in Germany, and 6% in the Netherlands [6]. For comparison, the current prevalence in Europe is 6% [5].

The development and spread of antibiotic resistance (AR) is correlated with the use of antibiotics in the healthcare sector and in the agriculture and husbandry sectors [1,3,7,8]. Antibiotic therapy is also a risk factor for carriage of AR by individuals. Other potential risk factors include: travels to high-risk areas for AR [2,9–28], consumption of food contaminated with AR bacteria [29,30], a poorer health status that leads individuals into being treated with antibiotics or at healthcare facilities increasing their exposure to AR bacteria [23,26], and occupation where the workplace might potentially increase exposure to antibiotics or AR bacteria, such as working at animal markets, dairy facilities, farms, slaughterhouses, wastewater treatment plants, and healthcare facilities [31–46]. However, most of the studies examining potential risk factors focus on high-risk populations, such as travelers [10,12,16,20,22,26,27,47], healthcare workers and patients [40,45,46,48–50], swimmers [51–53], farmers [33–36,38,39,41,43,44], and slaughterhouse workers [32], and often use small, convenient samples of, e.g., students [2,18,19,23]. However, risk factors for AR in the general population have not yet been sufficiently investigated. This is of great importance for developing preventive measures and antibiotic therapy policies.

As part of the larger AWARE study [6,54], this study aimed to explore, describe, and characterize potential risk factors for carriage of ESBL-EC in a large sample of European individuals recruited from the general population in three countries with a different prevalence of AR, i.e., Germany, the Netherlands, and Romania.

2. Materials and Methods

2.1. Study Design and Participants

The study population comes from participants enrolled in the large trans-European cross-sectional AWARE study (Antibiotic Resistance in Wastewater: Transmission Risks for Employees and Residents around Wastewater Treatment Plants). The full methodology of this project has been previously described [6,54]. The subset of the data used in these analyses corresponds to individuals from the general population living more than 1000 m away from a local WWTP, and, thus, not exposed to potential AR bacteria coming from such facilities. Data collection took place from September 2018 to March 2020 in Southern Germany, the Netherlands, and Romania. Having age between 16 and 67 years was an inclusion criterion.

In Southern Germany, we recruited participants using households as the unit of recruitment. We obtained household participant information from local civil registries. Invitation letters were mailed to all individuals older than 16 years of age within the household. For locations where we could not obtain participant information through the civil registries, invitation letters were dropped in household mailboxes by members of the study team. Aids in recruitment included two reminder letters, articles about the project in the local newspaper, recruitment campaigns via Facebook, and a raffle of shopping vouchers worth EUR 1500 in total for participants who completed the study. In the Netherlands, the offices of general practitioners served as recruitment points. We used ArcGis™ [55] to identify all postal addresses in a 500-m radius from 22 different General Practitioners' (GP) practices and then we randomly retrieved contact information for 200–500 households per GP practice using the Dutch Personal Records Database. The invitation to participate was addressed to all members of these households aged over the age of 16 (conform the conditions for General Data Protection Regulation data use). All participants who completed the study received a shopping voucher worth EUR 20. In Romania, we identified participant households and invited participants through door-to-door visits.

Ethics approval was obtained from the Ethics Committee of the University of Munich (LMU) (Project-No. 17-734) and the Research Ethics Committee of the University of Bucharest (Registration-No. 164/05.12.2017). The ethics board in the Netherlands exempted this study for ethical approval under the Dutch Medical Research Involving Human Subjects Act (WMO; Committee: Medisch Ethische Toetsingscommissie, number of confirmation: 19-001/C). All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki of 1975, revised in 2013.

2.2. Variables of Interest

Potential Risk Factors

Participants were asked to complete an online questionnaire [54] containing questions about socio-demographic characteristics, including date of birth (used to operationalize age in years), sex (female, male), educational level (according to the national educational system), and country of residence (Germany, the Netherlands, or Romania). The questionnaire also included questions about potential risk factors for carriage of ESBL-EC in the past year, such as: job history; hospital and farm visits (no, yes); contact with animals (no, yes); contact with patients or human tissues at work (never, rarely, sometimes, often, always); use of antibiotics and antacids (no, yes, do not know); self-reported health status (poor, fair, good, very good, excellent); self-reported frequency of diarrhea (never, rarely, sometimes, often, always); surgeries (no, yes); and international travel to Europe, Asia, North Africa, Sub-Saharan Africa, North America, Central America or Mexico, South America, and Australia or Oceania (never, once, 2–3 times, more than 3 times, do not know). The details on how these variables were chosen have been previously published [54].

Educational level was explored using national educational system levels and then dichotomized into low (pre-primary education to lower secondary education) or high educational level (upper secondary education to Doctoral or equivalent) according to the Standard Classification of Education (ISCED) [56–58]. Variables using a frequency scale with five levels were reduced to two levels in the case of frequency of diarrhea (never, rarely, or sometimes/often or always) and of self-reported health status (good, very good or excellent/fair or poor), and in the case of patient contact and of work with human tissues into three levels (never/rarely or sometimes/often or always). In questions including a "do not know" option (antibiotics and antacid intake, travels to Europe), this option was coded into the "no" category considering that the proportion of participants choosing this option was very low (3.1% for antibiotic intake, 2.9% for antacid intake, 0.1% for travels to Europe). We show descriptive counts for international travel variables as we collected the questionnaire data, i.e., using the following frequency scale for travel in the past 12 months:

"never", "once", "2–3 times", "more than 3 times", "do not know". For inferential analysis using regression models, these variables were collapsed into two levels: "never" and "at least once". For the regression models, travels to Central and South America were collapsed into one variable. Additionally, we constructed a travel score considering travel to Asia, North Africa, Sub-Saharan Africa, Central America or Mexico, South America, and the European countries Italy, Bulgaria, Greece, and Slovenia as high-risk areas for AR. The travel score adds one point for travelling once, two points for travelling 2–3 times, and 3 points for travelling more than 3 times to any of these areas in the past year, while "never" was translated into zero points.

2.3. Outcome of Interest

In the Netherlands, all recruited participants were asked to provide a stool sample using a stool sample kit. In Germany and Romania, only participants who completed the online questionnaire were asked to provide a stool sample. After sampling, stool samples were kept refrigerated, transported in cooling boxes (2 °C to 8 °C), and processed within 24 h. Samples were inoculated directly into TBX (only in the Netherlands and Romania) or MacConkey (in Germany) agar plates (for *E. coli)*, and on ChromID® ESBL (for ESBL-EC) and incubated at 36 °C ± 1 °C for 24–48 h. In case of positive results for ESBL-EC, 2 separate isolates per sample were collected from the selective ESBL plate for antibiotic resistance phenotype confirmation, and identification using MALDI-TOF MS (Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry). ESBL confirmatory tests were performed using cefotaxime and ceftazidime disks, alone and combined with clavulanate, following guidelines from the Clinical Laboratory Standards Institute (CLSI) [59]. The test was considered positive for strains showing a 5 mm increase in zone diameter in the presence of clavulanate. Stool sample results were coded binarily as positive or negative and included in the analyses.

2.4. Statistical Analyses

We used a Mann–Whitney test for observing differences in non-normally distributed numerical variables (age and travel score) and the Fisher's exact test for differences in proportions (all the other variables). Variable selection was performed using a combination of bivariate analysis results (p-value ≤ 0.2) and expert opinion. We regressed carriage of ESBL-EC on a set of potential risk factors using two logistic regression models. The first model included sociodemographic variables (age, sex, educational level, and country of residence), frequency of diarrhea, antibiotics use, and travel score. The second model was similar to the first one, except that, instead of the travel score, it included each geographical area as we assessed them in the questionnaire, with "Central America or Mexico" and "South America" collapsed into one variable. We report both crude and adjusted estimates for both models. Missing values were handled by multiple imputation where the missing mechanism was missing at random (MAR) or missing completely at random (MCAR). MAR means that the probability of the data being missing is not due to unobserved data, conditional on the data that were collected. MAR is the second-best scenario for multiple imputation after MCAR, which occurs when the probability of the data being missing does not depend on the observed or unobserved data, and is, thus, the best scenario for multiple imputation [60]. Multiple imputation diagnostic tables can be found in the Supplementary Materials (Supplementary Tables S1 and S2). Inverse probability of sample weights was used to adjust for non-response by country [61,62]. We present model results in odds ratios (OR) with the corresponding 95% confidence intervals (CI). All analyses were performed in R version 4.1.0 [63].

3. Results

3.1. Study Population

In Germany, we invited 3153 residents (response 11%), while in the Netherlands we contacted 13,918 identified individuals by postal service, of which 10,448 were eligi-

ble by age (response 6%), and in Romania we invited 280 residents (response 54%). A total of 1183 participants were included in the analyses: 333 from Germany, 689 from the Netherlands, and 161 from Romania. The average prevalence of ESBL-EC carriage across the three countries was 7.5%, which corresponds to 8.4% in Germany, 6.1% in the Netherlands, and 12.6% in Romania. A total of 109 participants (95 in Germany, 3 in the Netherlands, and 11 in Romania) did not hand in a stool sample or had non-valid stool samples (9.2%). The large proportion of missing stool samples in Germany stems from having a short window for sample collection and transportation in this location, with which many participants failed to hand in the sample. This, however, did not happen in the Netherlands or Romania where samples were to be brought to GP practices within a 500-m distance from people's homes collected by door-to-door visits.

The majority of participants in the overall sample were women (59.4%), middle-aged (median age 48 years, IQR 35–59), and highly educated (66.5%). Most participants reported no major risk factors for AR in the past year: no hospital visits neither as patient (92.9%), nor as professional (96.5%) or visitor (97.9%), no patient contact (73.6%), no use of antibiotics (76.1%) or antacids (77.2%), no surgeries (95.5%), no or infrequent diarrhea (94.2%), no work with human tissues (75.4%), no work with animals (96.5%), no work at a farm (99.0%), no work at a slaughterhouse (99.8%), no work with manure (97.0%), no farm visits (89.3%), and no animal contact (has no horses: 97.0%, has no dogs: 77.2%, has no cats: 75.7%). Additionally, most participants reported a health status from good to excellent (86.5%). Although a little more than two thirds of the study population reported travelling within Europe at least once in the past year (71.7%), they rarely traveled outside of the European continent: Australia or Oceania (1.0%), Central America (2.0%), South America (1.9%), Sub-Saharan Africa (2.4%), North America (3.6%), Northern Africa (4.2%), or Asia (7.2%). The proportion of population characteristics for individuals with a positive stool sample for ESBL-EC were similar as for the whole study population (Tables 1 and 2).

3.2. Risk Factors for ESBL-EC Carriage

Descriptive analyses including data from all study centers showed that ESBL-EC positive participants had higher education and were less likely to have a dog as a pet (Table 1). Furthermore, they were more likely to have had traveled at least once in the past year to Sub-Saharan Africa, Northern Africa, Asia, or North America according to bivariate analyses.

Country-specific analyses showed that travels to Northern Africa were associated with ESBL-EC carriage in the German sub-population, while an association was identified in the Dutch sub-population for traveling to Northern Africa, Sub-Saharan Africa, or Asia. In the Romanian subpopulation, high educational level, not having a dog as a pet, and working with human tissues were factors associated with ESBL-EC carriage. The travel score for travel to geographical areas with a known high-risk for AR, was significantly higher in the overall and Dutch ESBL-EC positive populations (p-value 0.02 and 0.001, respectively), compared to participants without ESBL-EC carriage (Table 2).

Confirming descriptive and bivariate results, self-reported travel to North Africa, Sub-Saharan Africa, and Asia at least once in the past year were identified as independent risk factors for ESBL-EC carriage in our study population, both in crude and adjusted models (Figure 1). A summary of the adjusted estimates for travel to different geographical areas can be seen in Figure 2.

Table 1. Categorical descriptive characteristics of ESBL-producing *E. coli* carriers by country, n = 1183.

			Overall, n = 1074		Germany, n = 238		The Netherlands, n = 686		Romania, n = 150	
Missing Values for Stool Samples, n			109		95		3		11	
Variable	**Missing**	**Level**	**ESBL_EC+, n (%)**	***p***	**ESBL_EC+, n (%)**	***p***	**ESBL_EC+, n (%)**	***p***	**ESBL_EC+, n (%)**	***p***
ESBL-EC positives			81 (8)		20 (8)		42 (6)		19 (13)	
Sex	4	Female	47 (7)	0.814	12 (9)	1.000	25 (6)	0.871	19 (13)	1.000
		Male	34 (8)		8 (8)		17 (6)		9 (13)	
Highest educational level obtained [a]	2	Low	19 (5)	**0.050**	6 (10)	0.602	13 (5)	0.196	0 (0)	0.217
		High	62 (9)		14 (8)		29 (7)		19 (14)	
Work with animals in the past year	35	No	75 (7)	0.752	18 (8)	1.000	40 (6)	0.659	17 (12)	0.555
		Yes	3 (8)		0 (0)		2 (8)		1 (17)	
Work at a farm in the past year	25	No	77 (7)	0.197	18 (8)	1.000	40 (6)	0.104	19 (13)	NA
		Yes	2 (18)		0 (0)		2 (22)		— (—)	
Work at a slaughterhouse in the past year	20	No	79 (7)	1.000	18 (8)	NA	42 (6)	1.000	19 (13)	1.000
		Yes	0 (0)		— (—)		0 (0)		0 (0)	
Work with manure in the past year	22	No	76 (7)	1.000	18 (8)	1.000	41 (6)	1.000	17 (12)	0.482
		Yes	2 (6)		0 (0)		1 (5)		1 (20)	
Patient contact or work with human tissues in the past year [b]	20	No	52 (7)	1.000	12 (8)	1.000	29 (7)	0.738	11 (10)	0.133
		Yes	26 (7)		6 (7)		13 (6)		7 (21)	
Patient contact in the past year	20	Never	58 (7)	0.481	13 (8)	0.552	31 (6)	0.872	14 (12)	0.672
		Rarely or sometimes	11 (9)		3 (12)		5 (7)		3 (18)	
		Often or always	9 (6)		2 (5)		6 (5)		1 (12)	
Work with human tissues in the past year	16	Never	57 (7)	0.704	13 (8)	1.000	32 (6)	0.928	12 (10)	0.097
		Rarely or sometimes	13 (9)		3 (8)		6 (7)		4 (25)	
		Often or always	8 (7)		2 (7)		4 (5)		2 (20)	
Hospital visits as a patient in the past year	0	No	76 (8)	0.672	16 (8)	0.517	41 (6)	0.507	19 (13)	0.597
		Yes	5 (6)		4 (11)		1 (2)		0 (0)	

Table 1. *Cont.*

			Overall, n = 1074		Germany, n = 238		The Netherlands, n = 686		Romania, n = 150	
Missing Values for Stool Samples, n			109		95		3		11	
Variable	**Missing**	**Level**	**ESBL_EC+, n (%)**	***p***	**ESBL_EC+, n (%)**	***p***	**ESBL_EC+, n (%)**	***p***	**ESBL_EC+, n (%)**	***p***
Hospital visits as a professional in the past year	0	No	78 (8)	0.761	19 (9)	1.000	41 (6)	1.000	18 (12)	0.336
		Yes	3 (8)		1 (5)		1 (7)		1 (33)	
Hospital visits as a visitor in the past year	0	No	79 (8)	0.690	18 (8)	0.169	42 (6)	1.000	19 (13)	1.000
		Yes	2 (9)		2 (22)		0 (0)		0 (0)	
Farm visits in the past year	4	No	71 (7)	0.578	17 (9)	0.773	35 (6)	0.097	19 (13)	0.596
		Yes	10 (9)		3 (7)		7 (11)		0 (0)	
Owning horses in the past year	139	No	77 (8)	0.722	20 (10)	0.605	40 (7)	1.000	17 (15)	1.000
		Yes	1 (4)		0 (0)		1 (7)		0 (0)	
Having dogs as pets in the past year	70	No	70 (9)	**0.011**	20 (11)	0.084	34 (7)	0.267	16 (16)	0.156
		Yes	9 (4)		0 (0)		7 (4)		2 (6)	
Having cats as pets in the past year	75	No	65 (9)	0.130	17 (10)	0.418	34 (7)	0.348	14 (15)	0.558
		Yes	13 (5)		3 (5)		7 (5)		3 (9)	
Use of antibiotics in the past year	0	No	60 (7)	0.685	13 (8)	1.000	36 (6)	0.544	11 (11)	0.289
		Yes	21 (8)		7 (8)		6 (5)		8 (17)	
Use of antacids in the past year	2	No	64 (8)	0.783	12 (7)	0.297	36 (7)	0.253	16 (13)	1.000
		Yes	17 (7)		8 (12)		6 (4)		3 (11)	
Surgeries in the past year	1	No	80 (8)	0.255	19 (9)	1.000	42 (6)	0.403	19 (13)	1.000
		Yes	1 (2)		1 (6)		0 (0)		0 (0)	
Self-reported frequency of diarrhea in the past year	4	Never, rarely or sometimes	74 (7)	0.223	17 (8)	0.069	38 (6)	0.347	19 (13)	1.000
		Often or always	7 (11)		3 (25)		4 (9)		0 (0)	
Self-reported health status in the past year	5	Good, very good or excellent	69 (7)	0.734	18 (8)	0.365	34 (6)	0.523	17 (13)	1.000
		Fair or poor	12 (8)		2 (13)		8 (7)		2 (11)	
Travel to high-risk areas for AR in the past year [c]	8	No	36 (6)	0.012	6 (6)	0.336	17 (4)	**0.004**	13 (13)	0.791

Table 1. *Cont.*

			Overall, n = 1074		Germany, n = 238		The Netherlands, n = 686		Romania, n = 150	
Missing Values for Stool Samples, n			**109**		**95**		**3**		**11**	
Variable	**Missing**	**Level**	**ESBL_EC+, n (%)**	***p***	**ESBL_EC+, n (%)**	***p***	**ESBL_EC+, n (%)**	***p***	**ESBL_EC+, n (%)**	***p***
Travels to Europe in the past year	5	Yes	42 (10)	0.498	13 (10)	0.378	24 (10)	0.718	5 (10)	0.498
		Never	27 (9)		5 (12)		11 (6)		11 (13)	
		Once	18 (8)		1 (2)		12 (7)		5 (18)	
		2–3 times	22 (6)		8 (8)		12 (5)		2 (8)	
		More than 3 times	12 (7)		5 (10)		7 (7)		0 (0)	
Travels to Bulgaria, Greece, Italy, or Slovenia in the past year	7	No	59 (8)	0.514	10 (8)	1.000	34 (6)	0.561	15 (15)	0.182
		Yes	19 (6)		9 (8)		7 (5)		3 (6)	
Travels to Sub-Saharan Africa in the past year	5	Never	73 (7)	**0.010**	19 (8)	1.000	36 (5)	**0.002**	18 (12)	NA
		Once	4 (19)		0 (0)		4 (22)		— (—)	
		2–3 times	1 (33)		— (—)		1 (33)		— (—)	
		More than 3 times	1 (50)		— (—)		1 (50)		— (—)	
Travels to Northern Africa in the past year	6	Never	69 (7)	**0.001**	17 (7)	**0.013**	35 (5)	**0.019**	17 (12)	0.324
		Once	8 (20)		2 (25)		5 (17)		1 (33)	
		2–3 times	2 (50)		1 (100)		1 (33)		— (—)	
		More than 3 times	0 (0)		— (—)		0 (0)		— (—)	
Travels to Asia in the past year	4	Never	63 (6)	**<0.001**	15 (7)	0.116	31 (5)	**<0.001**	17 (12)	0.408
		Once	13 (20)		3 (16)		9 (22)		1 (25)	
		2–3 times	2 (18)		1 (25)		1 (14)		— (—)	
		More than 3 times	1 (50)		— (—)		1 (50)		— (—)	
Travels to North America in the past year	4	Never	73 (7)	**0.036**	17 (8)	**0.041**	38 (6)	0.146	18 (12)	NA
		Once	5 (17)		2 (20)		3 (16)		— (—)	
		2–3 times	2 (25)		1 (50)		1 (17)		— (—)	
		More than 3 times	0 (0)		— (—)		0 (0)		— (—)	

Table 1. *Cont.*

			Overall, n = 1074		Germany, n = 238		The Netherlands, n = 686		Romania, n = 150	
Missing Values for Stool Samples, n			**109**		**95**		**3**		**11**	
Variable	**Missing**	**Level**	**ESBL_EC+, n (%)**	***p***	**ESBL_EC+, n (%)**	***p***	**ESBL_EC+, n (%)**	***p***	**ESBL_EC+, n (%)**	***p***
Travels to Central America or Mexico in the past year	6	Never	78 (7)	0.190	19 (8)	1.000	41 (6)	0.171	18 (12)	NA
		Once	0 (0)		0 (0)		0 (0)		— (—)	
		2–3 times	0 (0)		0 (0)		— (—)		— (—)	
		More than 3 times	1 (50)		— (—)		1 (50)		— (—)	
Travels to South America in the past year	6	Never	77 (7)	0.149	18 (8)	0.287	41 (6)	0.126	18 (12)	NA
		Once	1 (6)		1 (25)		0 (0)		— (—)	
		2–3 times	0 (0)		— (—)		0 (0)		— (—)	
		More than 3 times	1 (100)		— (—)		1 (100)		— (—)	
Travels to Australia or Oceania in the past year	6	Never	78 (7)	0.572	19 (8)	0.465	41 (6)	1.000	18 (12)	NA
		Once	1 (10)		1 (17)		0 (0)		— (—)	
		2–3 times	0 (0)		0 (0)		— (—)		— (—)	
		More than 3 times	— (—)		— (—)		— (—)		— (—)	

Notes: [a] Educational level according to the International Standard Classification of Education (ISCED): Low = ISCED 0–2 (Pre-primary education to Lower secondary education), High = ISCED $\geq$ 3 (Upper secondary education to Doctoral or equivalent). [b] Work with human tissues in the past year: Includes self-reported contact with human tissues (e.g., blood, urine, sputum, feces, vomit, saliva, or primary cell lines). [c] Travels to high-risk areas for AR in the past year: Includes travels to North Africa, Sub-Saharan Africa, Asia, Central and South America, as well as the European countries Italy, Greece, Bulgaria and Slovenia. ESBL_EC+: Positive stool sample for Extended-Spectrum Beta-Lactamase-Producing *E. coli*; AR: Antibiotic Resistance. Bold highlighting means statistically significant at the $p \leq 0.05$ level. Shown are the number of ESBL-EC carriers per variable and the percentage of ESBL-EC carriers relative to the total participants within the same level of that variable.

Table 2. Numerical descriptive characteristics of ESBL-producing *E. coli* carriers by country.

		Overall, n = 1074			**Germany, n = 238**			**The Netherlands, n = 686**			**Romania, n = 151**		
Missing Values for Stool Samples, n		**109**			**95**			**3**			**11**		
Variable	**Missings**	**ESBL_EC+**	**ESBL_EC−**	***p***	**ESBL_EC+**	**ESBL_EC−**	***p***	**ESBL_EC+**	**ESBL_EC−**	***p***	**ESBL_EC+**	**ESBL_EC−**	***p***
n		81	993		20	218		42	644		19	131	
Age, years (median [IQR])	0	47 [34, 57]	51 [37, 60]	0.172	38 [31, 50]	49 [36, 58]	0.146	55 [42, 61]	54 [39, 61]	0.964	39 [34, 44]	40 [33, 50]	0.739
Travel score (mean ± SD, median [min, max]) [a]	12	0.86 ± 1.60, 1 [0, 13]	0.46 ± 0.79, 0 [0, 15]	0.020	1 ± 0.94, 1 [0, 3]	0.64 ± 0.74, 1 [0, 6]	0.081	1.05 ± 2.07, 1 [0, 13]	0.42 ± 0.84, 0 [0, 15]	0.001	0.28 ± 0.46, 0 [0, 1]	0.38 ± 0.56, 0 [0, 3]	0.533

Notes: [a] Travel score was constructed based on frequency of personal travels to high risk areas for antibiotic resistance in the past year: Includes travels to North Africa, Sub-Saharan Africa, Asia, Central and South America, as well as the European countries Italy, Greece, Bulgaria, and Slovenia. The score is the sum of: zero points for not travelling to these areas in the past year, one point for travelling once to these areas in the past year, two points for travelling to these areas two or three times in the past year, and three points for travelling to these areas more than three times in the past year. Test used for bivariate hypothesis testing: Mann–Whitney test. ESBL_EC+: Positive stool sample for Extended-Spectrum Beta-Lactamase-Producing *E. coli*; ESBL_EC−: Negative stool sample for Extended-Spectrum Beta-Lactamase-Producing *E. coli*. IQR: Inter-quartile range. Bold highlighting means statistically significant at the $p \leq 0.05$ level.

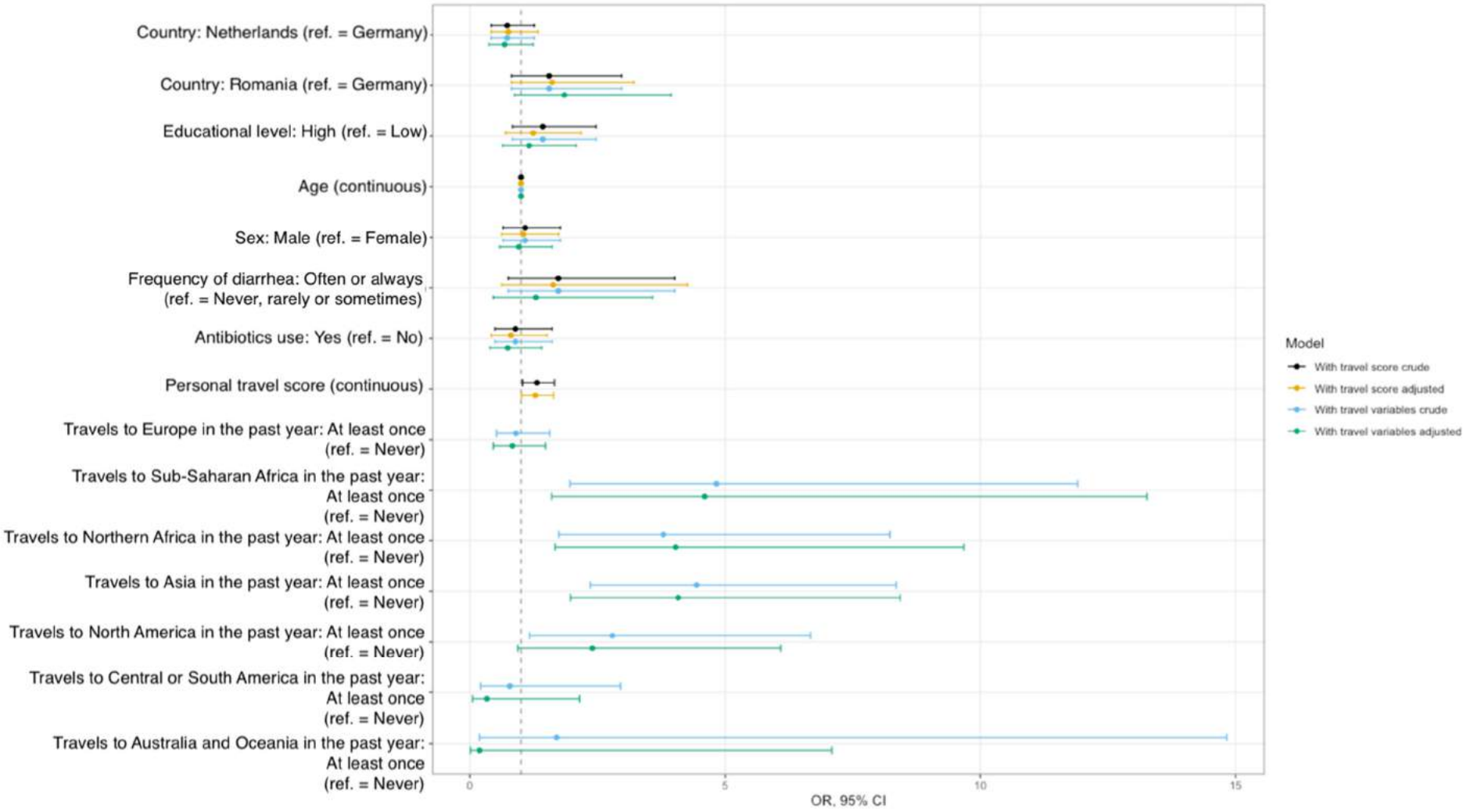

Figure 1. Risk factor analysis for carriage of ESBL-producing *E. coli* in stool samples.

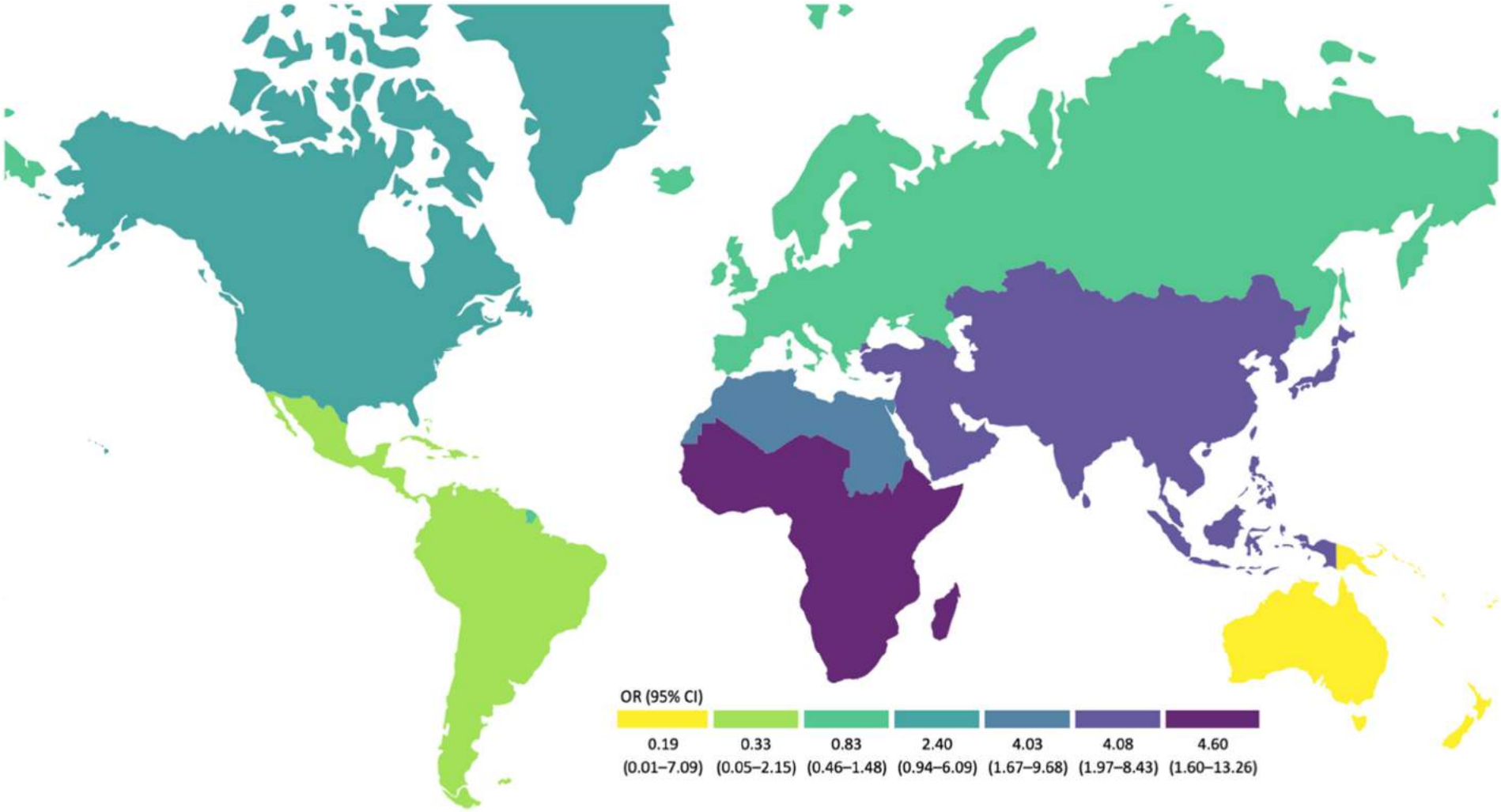

Figure 2. Travel areas as risk factors for ESBL-EC carriage (adjusted OR). Note: The European spot in South America corresponds to French Guiana.

On average, participants were about four times more likely to be carriers of ESBL-EC after travelling at least once in the past year to Northern Africa (adjusted OR 4.03, 95% CI 1.67–9.68), Sub-Saharan Africa (adjusted OR 4.60, 95% CI 1.60–13.26), and Asia (adjusted OR 4.08, 95% CI 1.97–8.43, Supplementary Table S4), compared with no travels to these regions. Although participants were twice as likely to be ESBL-EC carriers after traveling to North America, we could only identify a statistically significant association in the crude model (OR crude 2.79, 95% CI 1.17–6.67 vs. OR adjusted 2.40, 95% CI 0.94–6.09). The model including the travel score confirms these findings (Figure 1, Supplementary Table S3): Participants were 28% more likely to be ESBL-EC carriers when their travel score increased by one point, i.e., when they traveled at least once to any of the pre-specified high-risk areas for AR (adjusted OR 1.28, 95% CI 1.01–1.64, Supplementary Table S3).

4. Discussion

In this study, we found that destination for travels made during the past year is an important personal risk factor for carriage of ESBL-EC in the general population, especially North Africa, Sub-Saharan Africa, Asia, and—to some extent—North America. Other studies in risk populations have found similar results: some of these studies indicate that the prevalence of ESBL-EC acquisition is worryingly high in visitors returning from India, China and Southeast Asia, Middle East, Northern Africa, and Central and South America [64,65]. For European residents, travel outside of Europe was identified as a major travel risk factor [17]. A 2017 prospective study performed on Dutch travelers (n = 2001) found out that 34.3% of participants who were ESBL negative before travel, became positive for ESBL-EC during their travels, with the highest number being among participants travelling to Southern Asia [13].

We also found some differences in the country-specific travel patterns. By having collected a large sample size in The Netherlands, we were able to identify that this sub-population is at higher risk of ESBL-EC carriage when travelling to North Africa, Sub-Saharan Africa, and Asia within the past year. These results are comparable to those of a recently published large cross-sectional study of the Dutch general population, which identified traveling to Africa and Asia as independent risk factors for ESBL-EC carriage [66]. We found similar patterns in our German study population, where participants are at higher risk of ESBL-EC carriage after travels to Northern Africa and North America within the past year. Given that the national estimated prevalence of ESBL-EC causing urinary tract infections in the U.S. is 15.7%, ranging from 10.6% in the West North Central states to as high as 29.6% in the Mid-Atlantic states [67], our finding that travelers to North America were also at increased risk is not surprising. Conversely, in Romania, although the prevalence is already high, we found that the travel frequency is lower, therefore limiting our ability to analyze the effect of travel on ESBL-EC carriage in this subpopulation. Most of the Romanian participants reported not having travelled internationally at all within the past year. These findings suggest that the role of travel is country or context dependent.

The sewage surveillance data regarding the AR are in line with the estimated global burden of this threat. Current estimates indicate that the presence of AR genes found in the sewage is alarmingly at the highest level in Africa followed by Asia [68]. Models from sewage surveillance data show that the predicted clinical resistance to aminopenicillin, fluoroquinolones, and third generation cephalosporins are also at the highest resistance levels in Africa, followed by Asia [69]. These results from sewage surveillance data are in line with estimated global burden of disease from AR. The percentage of resistant isolates and the estimated death rate from AR *E. coli* have been reported to be at the highest in South Asia, followed by Sub-Saharan Africa [70]. Even though there have been some efforts in starting and maintaining clinical and sewage surveillance of AR bacteria in some countries of Africa and Asia [71], data on AR in these areas are still lacking to a large extent [70]. Some of these efforts include stewardship and surveillance programs in Ethiopia [72] and Ghana [73], or more generally in the African [74,75] and Asian regions [76–78]. The World Health Organization Global Antimicrobial Resistance and Use Surveillance System (WHO-GLASS)

Report in 2021 states that out of 47 African countries, territories, and areas, only half (23/47, 49%) are enrolled in GLASS and only a third (15/47, 32%) reported information from the national surveillance system to GLASS [79]. The South East Asia region provides a better outlook: out of 11 countries, territories, and areas in South East Asia, all of them are enrolled in GLASS, and nine of them (81%) reported information from the national surveillance system [79]. However, some of the challenges to these programs include bias in sampling and data collection in these areas, which leads to gaps in knowledge about the AR situation at the global level.

Our findings have implications for clinical practice. Asking patients about their travel history in the past year might help clinicians in their decision-making process for choosing specific antibiotic protocols as the first-, second-, or third-line of treatment. Further, the use of a travel score, such as the one we have constructed, might be a straightforward way of quantifying the degree of risk due to travel. However, our travel score is still far from ready to be used in clinical practice in its current form. On the one hand, it does not include other details about the travel experience, such as reason for travel, length of stay, or place of residence within the visited location. It might be that individuals who travel abroad for business reasons are exposed to a very different set of environmental factors than those who travel to visit friends or family, partly because their consumption patterns might be different. Additionally, closer interactions with locals might increase the risk of direct or indirect exposure to AR bacteria such as ESBL-EC when sharing toilets with friends or family members, as opposed to staying at a hotel with private toilet facilities and frequent cleaning and disinfection.

According to our data, no other risk factor explored besides travels posed an effect on carriage of ESBL-EC. Antibiotics use is a risk factor for AR commonly mentioned in the literature [23,26]. We believe that one of the reasons why we were not able to estimate an effect for antibiotics use in our study is that, although these effects are relatively easy to identify in high-risk populations such as travelers, farmers, slaughterhouse workers, healthcare providers, or patients, the sample size needed to detect an effect in the general population would be considerably higher. Another potential reason is that the effect of antibiotics use on AR might not be detectable more than 6 months after travel. A recent study by Bunt et al. [66] in 4177 Dutch participants from the general population (four times the size of our study) showed a positive effect of antibiotics use for ESBL-EC carriage up to 6 months before study participation, but not at 6 to 12 months, nor more than a year before participation.

The main strength of our study is that, to our knowledge, this is the first international study across several countries that confirms travel risks for AR in the general population. Whereas many previously published studies have indeed reported travel as a risk factor for ESBL-EC carriage, our study was performed on a large sample stemming from the general population. These are generally healthy, working adults that were recruited without considering any specific high-risk factor for AR. Yet, we have found that travel is a risk factor for carriage of ESBL-EC, have characterized high-risk geographical areas for travels, and have estimated the magnitude of the effect of travelling to these areas. Additionally, although the study population was enrolled as part of the large trans-European cross-sectional AWARE study, it was assumed that individuals from the general population living more than 1000 m away from a local WWTP were not exposed to potential AR bacteria coming from such facilities. Therefore, we have a relatively large sample of participants drawn from the general population in Southern Germany, the Netherlands, and Romania. In contrast, other similar studies explored risk factors in large sample sizes from only one country [66], in specific high-risk populations, such as farmers [33–36,38,39,41,43,44] and slaughterhouse workers [32], healthcare workers and patients [40,45,46,48–50], or travelers [8,10,14,18,20,25,26,46], or in convenient samples of students [2,18,19,23]. Further, when exploring frequency of travel, we considered all areas of the globe, and did not limit ourselves to low-and-middle income countries or other areas that would have been otherwise considered a priori as high-risk areas for AR.

Some of the limitations of our study include a low response, especially in Germany and in the Netherlands, and a high proportion of missing values, especially in Germany, which lead to a relatively low statistical power for some potential risk factors and might limit the representativeness of our sample. We have used analytical tools, such as inverse probability of sampling weights, based on the response and multiple imputation to address these issues. Our potential risk factors were assessed by a questionnaire instead of by direct measurement or by cross-referencing with medical data, which might lead to recall bias and, thus, misclassification based on the risk factors. If this was the case, we would be erring on the conservative side by underestimating potential effects. Further, our sample might not be exempt from selection effects as our population was relatively young and highly educated. Age and socio-economic status (SES) might also play a role in our estimation of results from travel variables because we might assume that younger people travel more often and to different regions of the globe than older people, or because people of a higher SES might have the financial resources and freedom to travel more often than people of lower SES. In our study, we have included age and educational level (as a proxy for SES) in our regression models, thus adjusting for these potential confounders.

5. Conclusions

In our study, we have identified travel to Northern Africa, Sub-Saharan Africa, Asia, and—to some extent—North America as independent risk factors for ESBL-EC carriage in a large sample of European individuals residing in Southern Germany, the Netherlands, and Romania. With our data, we were not able to identify other potential risk factors for carriage of ESBL-EC frequently mentioned in the literature such as the use of antibiotics within the past year, probably because the sample size needed to detect such effects in the general population would have to be at least about four times as large as ours. Further, we have developed a travel score that, although it needs refining to include information, such as reason for travel, length of stay, or place of residence, could be developed as a valuable tool in clinical practice when dealing with patients in need of an empirical treatment protocol with antibiotics. Questions about travel to Africa and Asia should continue to be routinely asked in clinical practice, as these travels are risk factors when considering antibiotic therapy.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijerph19084758/s1, Table S1: Multiple imputation diagnostics—Traditional (unweighted) logistic regression models, complete cases vs. imputed for model with personal travel score, AWARE Study, 2022; Table S2: Multiple imputation diagnostics—Traditional (unweighted) logistic regression models, complete cases vs. imputed for model with individual travel areas, AWARE Study, 2022; Table S3: Models comparing risk factors for ESBL-producing *E. coli* in stool samples, with personal travel score, AWARE Study, 2022; Table S4: Models comparing risk factors for ESBL-producing *E. coli* in stool samples, with individual travel areas, AWARE Study, 2022.

Author Contributions: Study conception and design: M.C.C., D.G.J.L., K.R., D.N., A.W., A.M.d.R.H. and H.S. Fieldwork and data collection: D.R.-M., H.B., M.P., M.K., L.M., G.P.G. and L.W. Microbiology: H.B., M.P., M.K., L.M., G.P.G., M.C.C., B.S., A.W., A.M.d.R.H. and H.S. Data cleaning and analysis: D.R.-M., H.B. and M.P. Interpretation of the data: D.R.-M., F.B., H.B., C.-F.F., L.W., D.G.J.L., K.R., A.M.d.R.H. and H.S. Drafting of the manuscript: D.R.-M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Ethics approval was obtained from the Ethics Committee of the University of Munich (LMU) (Project-No. 17-734) and the Research Ethics Committee of the University of Bucharest (Registration-No. 164/05.12.2017). The ethics board in the Netherlands exempted this study for ethical approval under the Dutch Medical Research Involving Human Subjects Act (WMO; Committee: Medisch Ethische Toetsingscommissie, number of confirmation: 19-001/C).

Informed Consent Statement: All subjects gave their informed consent for inclusion before they participated in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy reasons.

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5. Appendix 1

Wengenroth L, Berglund F, Blaak H, Chifiriuc MC, Flach CF, Pircalabioru GG, Larsson DGJ, Marutescu L, van Passel MWJ, Popa M, Radon K, de Roda Husman AM, **Rodríguez-Molina D**, Weinmann T, Wieser A, Schmitt H. Antibiotic Resistance in Wastewater Treatment Plants and Transmission Risks for Employees and Residents: The Concept of the AWARE Study. Antibiotics. 2021 Apr 21;10(5):478. doi: 10.3390/antibiotics10050478.

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Study Protocol

Antibiotic Resistance in Wastewater Treatment Plants and Transmission Risks for Employees and Residents: The Concept of the AWARE Study

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Abstract: Antibiotic resistance has become a serious global health threat. Wastewater treatment plants may become unintentional collection points for bacteria resistant to antimicrobials. Little is known about the transmission of antibiotic resistance from wastewater treatment plants to humans, most importantly to wastewater treatment plant workers and residents living in the vicinity. We aim to deliver precise information about the methods used in the AWARE (Antibiotic Resistance in Wastewater: Transmission Risks for Employees and Residents around Wastewater Treatment Plants) study. Within the AWARE study, we gathered data on the prevalence of two antibiotic resistance phenotypes, ESBL-producing *E. coli* and carbapenemase-producing *Enterobacteriaceae*, as well as on their corresponding antibiotic resistance genes isolated from air, water, and sewage samples taken from inside and outside of different wastewater treatment plants in Germany, the Netherlands, and Romania. Additionally, we analysed stool samples of wastewater treatment plant workers, nearby residents, and members of a comparison group living ≥1000 m away from the closest WWTP. To our knowledge, this is the first study investigating the potential spread of ESBL-producing *E. coli*, carbapenemase-producing *Enterobacteriaceae*, and antibiotic resistance genes from WWTPs to workers, the environment, and nearby residents. Quantifying the contribution of different wastewater treatment processes to the removal efficiency of ESBL-producing *E. coli*, carbapenemase-producing *Enterobacteriaceae*, and antibiotic resistance genes will provide us with evidence-based support for possible mitigation strategies.

Keywords: wastewater treatment plants; ESBL-producing *E. coli*; carbapenemase-producing *Enterobacteriaceae*; antibiotic resistance; employees; residents

1. Introduction

Antibiotic resistance has become a serious global health threat. As bacteria and certain genetic traits often move between humans, animals, and the environment, a one health approach that considers these interactions is needed to efficiently address this growing problem. The role of the environment in the emergence and dissemination of antibiotic resistance has become more and more acknowledged [1–3]. Still, little is known about the transmission dynamics of antibiotic-resistance determinants from water, air, and soil and their risks for humans in direct contact with these matrices [4]. A key to determining human health impacts lies in the application of epidemiological investigations, in which the carriage of antibiotic resistant bacteria (ARB) in people exposed to a specific transmission route is tested in comparison to unexposed or less exposed controls. Such studies have been carried out in travellers [5] and in agricultural settings [6,7], but other environmental exposure routes, such as via water, have rarely been studied [8–12].

Wastewaters from agriculture, industry, hospitals, and households are collected together at wastewater treatment plants (WWTPs), making them unintentional collection points for antimicrobials and ARB. Wastewater typically harbours a mix of residual antibiotics and other agents that are known to co-select for antibiotic resistance [13,14], which provides opportunities for selection of ARBs and hence risks for evolution and transmission of resistance. Selection pressures, together with a high density and diversity of pathogens and environmental bacteria carrying various antibiotic resistance factors, provide a milieu where new forms of resistance may emerge [15,16]. From mining of metagenomics data, we know that emergence of new antibiotic resistance genes (ARG) occurs [17,18]. Additionally, resistant bacteria already present in human faeces can pass WWTPs. For example, ESBL-producing *E. coli* (ESBL-EC) have been detected in the influent and effluent of WWTPs and the receiving surface waters [19]. It is known that human infections with ESBL-EC or carbapenem-resistant *Enterobacteriaceae* (CPE) are associated with increased mortality rates, time to effective therapy, length of hospital stay, and overall healthcare costs [20].

WWTPs are in general not developed to remove either of these (or any) resistant bacteria. Studies indicate that even though a significant reduction occurs through various treatment processes [21], significant amounts of antimicrobials, ARB, and ARGs are still shed into environmental reservoirs, including rivers and recreational water [22]. While the efficiency of conventional treatment technologies greatly differs between types of WWTPs, the role of specific treatment technologies in removal of antimicrobials, ARB, and ARGs remains poorly described [23,24].

Workers at WWTPs are potentially exposed to wastewaters carrying ARB and ARGs and aerosolised ARB and ARGs through different transmission routes: inhalation, dermal contact, and ingestion. Airborne bacteria have indeed been detected in WWTPs [25–27], including *Enterobacteriaceae* and faecal coliforms [28,29], and an increased prevalence of gastrointestinal and respiratory diseases was reported in WWTP workers, suspected to be linked to microbial exposures [30]. Although few studies so far addressed specific pathogens in WWTP workers, one has found an elevated carriage of *Tropheryma whipplei* [31]. Additionally, a higher seroprevalence of IgG against *Helicobacter pylori* was observed among sewage workers [32]. Hepatitis A virus, hepatitis E virus and positive stool PCR tests for *Leptospira spirochete* [33] were also described. However, the carriage of ARB and ARGs in WWTP workers is yet unknown.

Furthermore, WWTPs are often located in urban settings in close proximity to residents. As bacteria can be traced back up to 150 m away from animal farms [34], neighbouring residents might also face a risk of exposure to aerosolized wastewater. WWTPs, their workers, and nearby residents therefore could represent an ideal—but yet unstudied—test

case to investigate whether transmission via (waste) water actually impacts ARB and ARGs carriage.

Within the AWARE study (Antibiotic Resistance in Wastewater: Transmission Risks for Employees and Residents around Wastewater Treatment Plants), we gather data on two antibiotic resistance phenotypes, i.e., ESBL-producing *E. coli* (ESBL-EC) and carbapenem-resistant *Enterobacteriaceae* (CPE) and on ARG prevalence from analysis of air, water, sewage, and stool samples taken from inside and outside of different WWTPs in Germany, the Netherlands, and Romania. The AWARE study specifically aims:

1. To study carriage rates of ESBL-EC, CPE, and of a range of clinically relevant ARGs in WWTP workers and nearby residents (living within ≤300 m vicinity of a WWTP) compared to a comparison group (living 1000 m away from the closest WWTP);
2. To study waterborne and airborne exposure to ESBL-EC, CPE, and of a range of clinically relevant ARGs in WWTP workers through ingestion and inhalation;
3. To assess the efficiency of different WWTP treatment technologies in diminishing ESBL-EC, CPE, and a range of clinically relevant ARGs; and
4. To investigate selection and emergence of ESBL-EC, CPE, and a range of clinically relevant ARGs in WWTPs through studying relative changes in resistance genes and exploring putative novel resistance genes from metagenomics data.

Our overall aim with this methodological publication is to deliver precise information about the methods used in the AWARE project, including selection of participants, sample taking, creation of the questionnaire, and pilot study. This publication is a study protocol which is purely methodological and does not include results of the study. Further, we will discuss possible strengths and limitations of our study design.

2. Materials and Methods

2.1. Study Design

The AWARE study is a multicentre, cross-sectional study investigating the prevalence of ESBL-EC, CPE, and ARGs in WWTP workers, residents living within ≤300 m vicinity of a WWTP (residents), and a comparison group living >1000 m away from the closest WWTP (comparison group). The field phase is carried out in Germany (DE), the Netherlands (NL), and Romania (RO) (Figure 1.).

2.2. Study Population

We aim to include 450 WWTP workers (150 per country). In order to compare carriage of ESBL-EC, CPE, and ARGs, we aim to include 800 nearby residents (400 in DE, 400 in RO) living in <300 m vicinity of a WWTP (residents). Further, we aim to include 1200 residents (400 in DE, 400 in RO, 400 in NL) living >1000 m away from the closest WWTP (comparison group). Assuming an average ESBL-EC prevalence of 8% in the general population, this would allow us to detect a minimum odds ratio (OR) of 1.7 with power 80% in workers and nearby residents on a 5% significance level.

In order to be included in the study, participants have to be within the age range of 16 to 67 years. All participants who have worked at a slaughterhouse or a farm during 12 months prior to study are excluded because contact with farm animals and working at slaughterhouses can be risk factors for ESBL-EC carriage [35].

2.3. Recruitment Process

The recruitment process for WWTP workers, residents living within ≤300 m of a WWTP, and the comparison group consisting of residents living >1000 m away from the closest WWTP is underlying local regulations and thus differs between DE, NL, and RO (Table 1). However, to control for seasonal variation of ESBL-EC, CPE, and ARGs, we aim to take all samples (water, air, stool) from the surroundings of each WWTP within eight weeks.

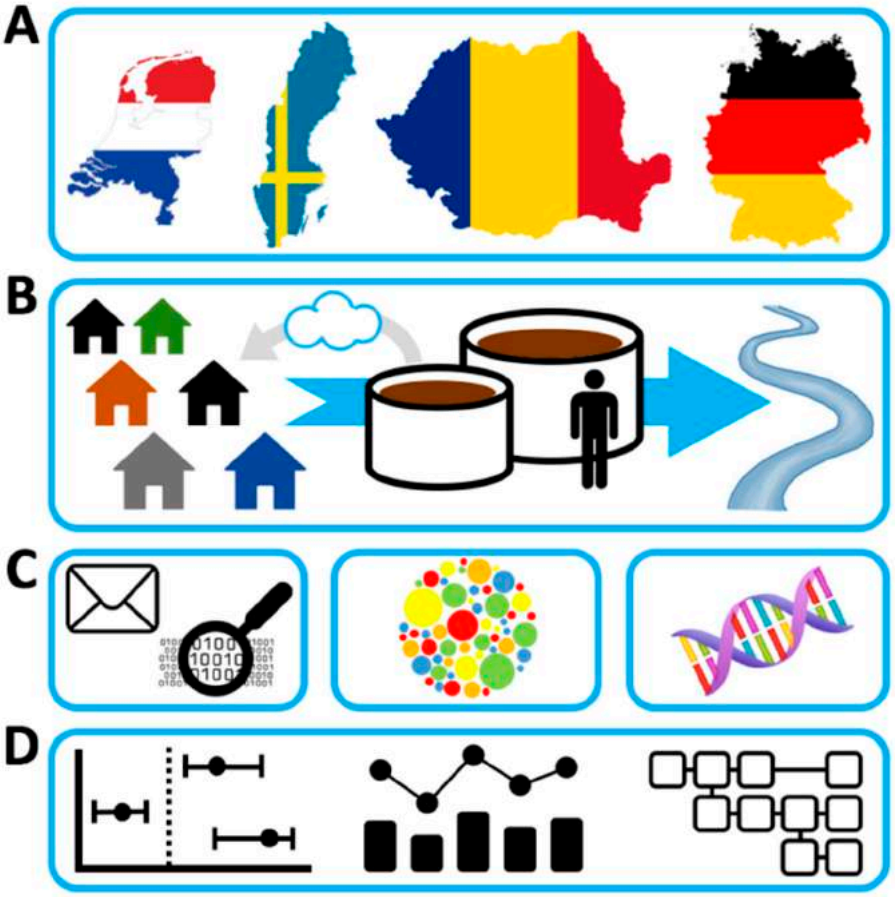

Figure 1. Legend Graphical Abstract: Overview of AWARE study, with (**A**) the participating countries, (**B**) the study domain, wastewater treatment plant samples, and workers of and residents living nearby wastewater treatment plants, (**C**) the techniques involved (questionnaire, molecular and cultural analyses of ESBL-EC, CPE, and the resistome, and (**D**) the outcome: epidemiological evaluation of differences in prevalence of ESBL-EC, CPE, and the resistome between workers and residents of wastewater treatment plants and the general population, changes in relative and absolute resistance along different wastewater chains, and models for airborne and waterborne exposure to resistant bacteria and resistance genes.

Table 1. Recruitment of participants into the AWARE study.

	Germany	The Netherlands	Romania
Selection of WWTPs	Eligible WWTPs are selected due to the following criteria: There are residents living in <300 m vicinity of WWTP, WWTP is located close enough to laboratories for the analyses of samples	All 21 regional waterboards [3] are included.	WWTPs are chosen to assure a good representativeness of different regions across the country.
Invitation of WWTPs	The operators of the WWTPs are contacted by the local study team and asked to participate.	The waterboards are informed of the study through the Dutch Water Authorities and asked to participate.	The operators of the WWTPs are contacted by the local study team and asked to participate.
Response in WWTPs	8 WWTPs are interested in participating.	12 waterboards are interested in participating [4].	9 WWTPs are interested in participating.

Table 1. *Cont.*

	Germany	The Netherlands	Romania
Study presentation and informing of WWTP workers	The study team visits 6 interested WWTPs and presents the project to the workers [1].	The WWTP workers of 10 waterboards are invited to attend a presentation of the study by the local study team [5]. The workers of the remaining 2 waterboards are recruited internally through email. By sending the presentation to all workers via email, also workers not attending the meeting are reached.	The WWTP operators inform and invite the employees to participate. Afterwards, several short information sessions are organized at the WWTPs for recruiting participants.
Informing of nearby residents	The study team researches the street names of all streets within ≤300 m vicinity of a participating WWTP through Google Maps and asks the local registration office [2] for the full address of all persons aged 16–67 years and having their main residence in those streets.	Due to concerns of the waterboards, residents living in ≤300 m vicinity of a WWTP cannot be included.	Invitations to the study are done using door-to-door approach. Additionally, in public places like streets, parks, and markets, potential participants are orally addressed and information sheets with details about the study are distributed. The participants are at least 18 years old.
Informing of comparison group	The addresses are collected in the same way as for the nearby residents, except that addresses >1000 m away from the closest WWTP and close to a train station are chosen to allow fast transportation of samples by the study team.	All addresses within a 500 m radius of GPs, who are willing to cooperate, are identified [6]. Then, 300–500 addresses per GP are randomly selected to extract personal data from the Dutch Personal Records Database (BRP). Information on the study is sent to all residents living at the selected addresses over 16 years of age.	Same procedure as for nearby residents
Incentives for participants [7]	Participants participate in a raffle with 10 shopping vouchers with a total value of 1500 Euros.	Every participant receives a gift card worth 20 Euro.	Every participant is granted 5 Euro.
Timing of sample taking	To control for seasonal variation of ESBL-EC, CPE, and ARGs all samples (water, air, stool) from the surroundings of one WWTP are aimed to be taken within eight weeks.		

[1] Two WWTPs stepped back from participation because they feared that residents and media might complain about WWTPs in case ESBL-EC, CPE, or ARGs would be found in their WWTP. [2] If addresses cannot be retrieved from the local registration offices, members of the study team go from door to door to recruit participants. In case of no reply, up to two reminders are sent (7 and 21 days after initial invitation). Further methods will be performed to increase the response: newspaper articles describing the AWARE project published by local newspapers, online advertisement on the study's Facebook page and in groups like notice boards and job advertisements, flyers about the AWARE study in doctors' offices of local physicians, invitations via e-mail to workers from different work fields (industry and public sector). [3] Waterboards are regional government bodies supervising, e.g., sewage treatment in their respective regions. [4] Nine waterboards did not want to participate out of fear for causing commotion among nearby residents or workers, or lack of interest to invest time and/or manpower to help organize recruitment. [5] WWTP workers generally work at multiple WWTPs, making it impossible to study workers of specific WWTPs. Therefore, all workers of waterboards were invited to participate, but only a selection of WWTPs (1–3 per waterboard) are selected for environmental sampling. [6] General practitioners (GP) within a 2–5 km distance from selected WWTPs are approached for cooperation, to function as a collection and preservation point of stool samples. Addresses of within a 500 m radius of GPs are identified using Geographical Information System (GIS) software (version ArcGis 10.6.1). [7] Participants who hand in a stool sample and a completed questionnaire.

2.4. Pilot Study

We test the study methods in a pilot phase which includes recruitment of study participants, the study questionnaire, and sample taking (water, air, stool). The study questionnaire is tested by 33 participants. Fifteen participants hand in stool samples for the pilot study. Additionally, all six water and sludge samples are taken from two WWTPs.

2.5. Study Instruments

2.5.1. Study Questionnaires

WWTP workers, residents, and members of the comparison group willing to participate receive access to an online questionnaire. However, we offer paper questionnaires at the preference of the participants. For quality control, we do double data entry with error check. The questionnaire assesses socio-demographics (age, gender, education) as well as potential risk factors for ESBL-EC, CPE, or ARGs carriage (work history, travel abroad, contact with farm animals, hospital visits, antibiotic intake, self-evaluation of general health condition). Additionally, WWTP workers also answer questions considering their specific work tasks at the WWTP, the use of personal protective equipment, and hygienic behaviour. WWTP operators answer questions about the capacity of their WWTP, origin of treated wastewaters, and wastewater treatment methods.

Whenever possible, we retrieve questions from validated questionnaires [36–45]. Only if we cannot find validated questions, we take items from existing, but not validated, questionnaires after checking for their face validity. If we cannot find any suitable questions from previous studies, we create expert validated new items. We translate the original questionnaires from English (Supplementary S1–S3) to German, Dutch, and Romanian. At least two experts on the topic who are also native speakers of the target language check the translation and provide feedback. This pre-pilot phase of the study is an iterative process to translate, back-translate, ask for feedback, and improve the current version of the questionnaires. We then test the translated questionnaires in a two-phase procedure: in the first phase, we recruit a small number of participants (n = 3) to read and provide verbal feedback on their understanding of each question. As we offer the questionnaire online, we create an online survey using LimeSurvey [46]. In the second phase, three persons of the target group go through the process of filling out the questionnaires online. They also provide feedback on the understanding of each question, and the online survey's functionality. Once the questionnaire is refined and tested for clarity and understandability, it is tested in the pilot study. During the pilot study, seven WWTP operators (one from DE, six from RO) and twelve WWTP workers (three from DE, nine from RO), two nearby residents, and twelve members of the comparison group fill in the questionnaire and provide feedback. Based on the results, we refine the questionnaire.

2.5.2. Stool Samples

In DE and RO, participants receive a stool sample kit by postal service (residents and members of the comparison group) or at work (WWTP workers) after handing in an informed consent and completing the questionnaire. In NL, participants first hand in their stool samples and then fill in the questionnaire. We provide all necessary material to the participants in order to take the stool sample. This includes a paper faeces collection device, a sterile stool sampling tube, and written and drawn instructions. In DE, participants are asked to bring the stool sample directly to the next WWTP, where it is cooled or stored temporarily in a refrigerator until the next morning, when it is collected by a member of the study team. In NL, we ask participants working at a WWTP to bring their stool sample to the WWTP, where it is cooled, while residents are asked to bring it to a specified general practitioner (GP). GPs within a 2–5 km distance from selected WWTPs are approached for cooperation, to function as a collection and preservation point of stool samples. Addresses of within a 500 m radius of GPs are identified using Geographical Information System (GIS) software (version ArcGis 10.6.1). Participants who are unable to bring their sample to the GP at the indicated time/day are given the opportunity to send the samples per mail

without cooling (although samples shipped per mail will be excluded from metagenomic sequencing). In RO, we ask participants to cool the stool samples at 1–10 °C directly after samples were taken and to bring them to the WWTP the next day. The same day, the stool samples are transported to the laboratory and processed within 72 h. We tested this procedure in the pilot study with fifteen participants (one WWTP operator, three WWTP workers, and eleven members of the comparison group).

At the local laboratories in DE, NL, and RO, all stool samples are inoculated directly onto the following agars: TBX or MacConkey, ChromID ESBL, ChromID OXA-48, and ChromID CARBA and incubated at 36 ± 2 °C for 24–48 h. In case of positive results, a total of two isolates belonging to the ESBL-EC phenotype and 5 isolates belonging to CPE phenotype are collected, screened for antibiotic resistance and identified by MALDI-TOF MS (Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry). We then process stool samples for DNA isolation after intermediate storage at −80 °C, which we then will use for subsequent metagenomics and qPCR analyses.

2.5.3. Water Samples

We collect water samples from WWTPs at four different treatment stages: wastewater influent (WI), effluent (WE), liquid sludge from the main biological reactor (e.g., aeriation tank) (AT), and dewatered sewage sludge after thickening (S). We also take water samples from the receiving surface water 200 m upstream (WU) and 200 m downstream (WD) of the WWTP. The following Figure 2 provides an overview of the collection points of water sample, as well as stool and air samples taken. We tested this procedure in the pilot study at one WWTP in DE and two in RO.

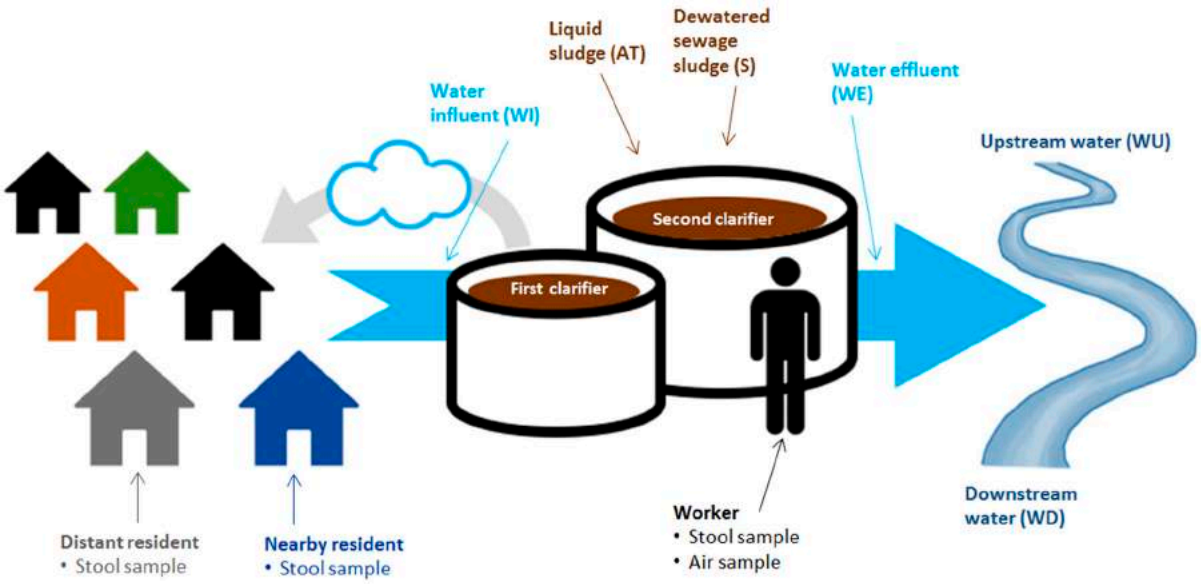

Figure 2. Collection points of water, air, and stool samples.

We collect upstream (WU) and downstream (WD) water samples as close as possible to the WWTP to minimize the influence of other sources, but at enough distance to minimize the chance of diffusion to upstream locations and to ensure sufficient mixing with effluent for downstream locations. If accessible, we choose locations at 200 m upstream and 200 m downstream for waters with a width <20 m, according to the rule of thumb that complete mixing occurs at a distance of at least 10× the width of the surface water. Additionally, we choose the upstream and downstream locations in a way that no additional side streams enter the river between these locations and the effluent discharge point. Therefore, we choose locations closer to the WWTP when side streams are present within the optimal distance. We take subsurface samples according to international guidelines (ISO 19458:2007: Water quality—Sampling for microbiological analysis).

The sampling points for wastewater influent (WI) and effluent (WE) are determined by the location of the flow-proportional auto samplers at the individual WWTPs, when present. Influent samplers are usually located directly after mechanical treatment and effluent samplers after completion of treatment, prior to discharge. Using auto samplers, experienced WWTP or laboratory staffs collect 24-h flow proportional samples, of which 1 L is transferred to a sterile bottle at the end of the usual time interval applied in the WWTP (e.g., 9:00 in the morning). If no automatic samplers are available, we take grab samples from wastewater influent and effluent, at approximately 40 to 60 percent of the water depth, at a site with maximal turbulence to ensure good mixing and the possibility of solids settling is minimized. The most desirable sampling locations for grab samples of influent include: (a) the upflow siphon following a comminutor (in absence of grit chamber); (b) the upflow distribution box following pumping from main plant wet well; (c) aerated grit chamber; (d) flume throat; (e) pump wet well when the pump is operating; or (f) downstream of preliminary screening.

When possible, we take influent samples upstream from side stream returns. We collect grab samples of effluent at the site specified in the sampling plan, or if no site is specified, we select the most representative site downstream from all entering wastewater streams prior to discharge into the receiving waters.

We take the liquid sludge sample (AT) from the main biological reactor (e.g., aeration tank). The selection of the sampling points depends on (a) the practicality of interrupting safely a stream of moving liquid sludge or cake when manually sampling; and (b) the nature of the chamber or tank design with respect to stratification of liquid sludges.

We take the sample of dewatered sewage sludge after thickening (S). Prior to the proposed sampling date, we assess sludge processing (dewatering and treatment) to ensure that sludge is in the appropriate form (liquid versus dewatered, untreated cake versus treated biosolids) and is available for sampling at the proposed date, time, and sampling point. If needed, we will adjust the selection points.

After all water and sludge samples are collected, they are kept at 1–10 °C at the WWTP and transported at 1–10 °C to the laboratory in NL (samples from DE and NL) and RO (samples from RO). At the laboratories in NL and RO, we process all samples within 48–72 h after sampling, e.g., homogenization (for sludge) and membrane filtration (for sludge and water). We then process water filters for DNA isolation, which we use for subsequent metagenomics and qPCR analyses.

2.5.4. Air Samples

We intend to ask a subset of 50 workers from 10 WWTPs per participating country to collect air samples to analyse personal exposure. Sampling is based on GSP inhalable sampling heads equipped with Teflon filters on Gilair pumps (3.5 L/min), sampling the total inhalable air of workers whose job position included activities at different treatment stages.

The pumps are programmed and fixed at the worker's belt or pocket by a member of the study team. A study team member checks the correct functioning of the pumps at the beginning, after three hours, and after six hours of sampling. After six hours, the study team member turns off the pumps. We wrap the heads of the pumps in aluminium foil and transport them directly to the laboratory where the pumps are opened on a sterile work bench. The laboratory assistants remove the Teflon filters with a pair of sterile tweezers and freeze them at −20 °C (DE) or −80 °C, respectively (NL, RO). We ship all filters to NL for analysis. Feasibility of the procedures is checked during the pilot study.

2.6. Metagenomic Analysis

The Swedish and Romanian team conduct culture-independent analyses. They will employ shotgun metagenomics sequencing [47–49] by the Illumina NovaSeq technology. This enables simultaneous quantification of any known antibiotic-resistance gene if present at sufficiently high levels to allow detection. In addition, shotgun metagenomics allows

for the analysis of mobile genetic elements such as integrons and transposons and of the taxonomic composition of the microbial communities [49]. Although costs for DNA sequencing have dropped dramatically, it still involves substantial costs if relatively rare resistance genes are targeted in complex community samples [48,50]. Therefore, we will select a subset of air, sewage, water, and faecal samples for sequencing, while we plan to choose 24 genes for qPCR investigations in all human, water, and air samples. The selection will be based on an initial screen using qPCR arrays with considerably more genes for a subset of samples. Antibiotic residues and their metabolites are usually detected in the environment at trace levels but may still be present at concentrations that have the potential to select for microbial resistance [49,51] and possibly also induce horizontal gene transfer [52]. Therefore, residues are monitored by high-performance liquid chromatography interfaced with tandem mass spectrometry (HPLC–MS/MS) in selected plants, including the WWTPs in which metagenomics data are also determined. We perform sample selection for metagenomic analyses by using propensity score matching of the exposed and unexposed groups to achieve proportional and non-statistically significant balance of the groups at a 5% statistical level.

2.7. Data Management

We store the personal contact data of participants and the history of contacts via letters, e-mails, and phone calls in a password protected Access database separated from questionnaire and sample data. We pseudonymize all assessed data. The laboratories document results of stool, air, and water samples in Excel. We primarily do data cleaning and analysis in R. Additional software will be used depending on the specific analyses. All personal data are stored password protected with access only to the members of the study team. We ensure that data management is bound to FAIR principles [53], e.g., including storage of research data obtained in publicly accessible and findable repositories.

2.8. Statistical Analysis

For descriptive analyses we assess the distribution of numerical variables visually for normality using histograms and present the mean ± standard deviation if normally distributed or the median ± inter-quartile range if non-normally distributed. We present categorical variables using absolute and relative frequencies. We handle missing values by multiple imputation in case of missing at random or missing completely at random. We do data cleaning, as well as multiple imputation, propensity score matching, data presentation, and outcome models using the statistical software R version 3.5 and up [54]. Additional software will be used and documented depending on the specific analyses.

We perform bivariate hypothesis testing choosing an appropriate statistical test depending on the type of variables involved, their distribution, and the number of counts per cell (for categorical variables). We perform logistic crude and adjusted regression models for the main outcomes such as carriage of ESBL-EC, CPE, and ARGs. Main exposure variables will include whether a participant belongs to the group of WWTP workers, nearby residents, or the comparison group. We consider linear regression models for secondary outcomes if these are numerical. We present results from regression models with the point estimate and its corresponding 95% confidence interval. We do variable selection for the models using a combination of experts' opinion from within the AWARE consortium, evidence in the current literature, and the use of Directed Acyclic Graphs (DAGs).

3. Discussion

To our knowledge, this is the first study investigating the potential spreading of ESBL-EC, CPE, and ARGs from WWTP to workers, the environment, and nearby residents. By involving different European countries, covering a variety of different types of WWTPs, our results will be relevant for a large number of situations. The methodological combination of epidemiology, molecular biology, and metagenomics will allow us to draw multilevel conclusions. We demonstrated feasibility of the AWARE project in the pilot study.

Our study is carried out cross-sectionally at each WWTP. Thus, the study does not provide information how the numbers of ESBL-EC, CPE, and ARGs vary with time/seasons. It is possible that bias arises for some samples due to different laboratories analysing them. In order to minimize such biases, we develop all SOPs jointly and centralize sample preparation and analyses whenever possible. WWTP workers are organized in different ways depending on the country: In NL, WWTP workers do not work at one specific WWTP, hampering the comparison between ESBL-EC, CPE, and ARGs at the selected WWTP and in stool from workers.

Our assessment of transmission of antibiotic-resistant bacteria from WWTPs to the surrounding environment will enable us to formulate recommendations, such as adapted sewage treatment, or recommendations for a minimal distance between WWTPs and residential buildings in order to reduce transmission of antibiotic resistant bacteria.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/antibiotics10050478/s1, Supplementary S1: AWARE questionnaire workers, Supplementary S2: AWARE questionnaire operators, Supplementary S3: AWARE questionnaire residents.

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