

Epidemiological and microbiological characteristics of *Salmonella* spp. infections in outpatients in Split-Dalmatia County in 2022: a cross-sectional study

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**EPIDEMIOLOGICAL AND MICROBIOLOGICAL CHARACTERISTICS OF
SALMONELLA SPP. INFECTIONS IN OUTPATIENTS IN SPLIT-DALMATIA
COUNTY IN 2022: A CROSS-SECTIONAL STUDY**

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LIST OF ABBREVIATIONS

ACN – Alert and Cooperation Network

AMC, AUG – amoxicillin-clavulanate

AMP – ampicillin

AMR – antimicrobial resistance

ARM – acid-resistance mechanism

C – chloramphenicol

CAZ – ceftazidime

CDC – Centers for Disease Control

CFR – case-fatality rate

CIA – critically important antimicrobial

CIP – ciprofloxacin

CRE – carbapenem-resistant *Enterobacteriaceae*

CRO – ceftriaxone

ECDC – European Center for Disease Control

EEA – European Economic Area

EFSA – European Food Safety Agency

ESBL – extended-spectrum beta-lactamases

EUCAST – European Committee on Antimicrobial Susceptibility Testing

FBD – Foodborne disease

FBO – foodborne outbreak

FERG – Foodborne Disease Burden Epidemiology Reference Group

HAH – The Croatian Food Agency

HGT – horizontal gene transfer

HIV – human immunodeficiency virus

hpCIA – highest-priority critically important antimicrobial

HR – hospitalization rate

H₂S – hydrogen sulfide

iNTS – invasive non-typhoidal *Salmonella*

ISKRA – Croatian Interdisciplinary Section for Antibiotic Resistance Control

JNS – Joint Notification Summary

KIA – Kligler's Iron-agar

LPS – lipopolysaccharide

MCO – multi-country outbreak

MDR – multi-drug resistance

MLVA – Multiple-Locus Variable-Number Tandem Repeat Analysis

NR – notification rate

NTS – non-typhoidal *Salmonella*

OB – outbreak

PMQR – plasmid-related quinolone resistance

PPI – proton pump inhibitor

RASFF – Rapid Alert System for Food and Feed

ROA – Rapid Outbreak Assessment

SDC, SDŽ – Split-Dalmatia County

ST – sequence type

SXT, TS – trimethoprim-sulfamethoxazole

TESSy – The European Surveillance System

UHS – University Hospital of Split

WGS – whole-genome sequencing

WHO – World Health Organization

XLS – Xylose-Lysine-Deoxycholate

1. INTRODUCTION

1.1. Genus *Salmonella*

The worldwide burden of foodborne disease (FBD) is immense, with the World Health Organization (WHO) estimating 550 million cases and 30 000 deaths from FBD each year (1,2). Up to 70% of all FBD globally, as well as thousands of deaths, are thought to be caused by *Salmonella*, or rather, members of genus *Salmonella*, one of the many genera in the family *Enterobacteriaceae*, order *Enterobacteriales*, class *Gammaproteobacteria*, phylum *Pseudomonadota*, consisting of gastrointestinal, mostly zoonotic, rod-shaped pathogens (3,4). According to the modern classification used by organizations such as the World Health Organization (WHO) and the Center for Disease Control (CDC), there are two species of *Salmonella*: *Salmonella bongori* and *Salmonella enterica*, the latter of which has six (I-VI) currently recognized subspecies (5,6). Within these subspecies, there are over 2500 serotypes, or serovars, as referred to by the WHO and the Pasteur Institute, each of which may have several different strains (7). Serotypes are typically classified according to the system known as the Kauffman-White-Le Minor schema, based on serotype-specific surface antigen patterns denoted by antigenic formulae, which is being increasingly challenged by DNA and genome-based methods of classification (7-11).

The nomenclature of *Salmonella* spp. variants is a complex and historically controversial subject; names of different variants, like *Salmonella subsp. enterica* serotype Enteritidis – though this is considered incorrect by some – are often omitted in favor of writing the variant's name as one would a species in a genus: *Salmonella* Enteritidis (4). Unless specified otherwise, the *Salmonella* variants mentioned in this paper are assumed to be of the subspecies *enterica*, which contains nearly all human pathogenic variants (12). As such, the serotypes discussed will be referred to for convenience's sake as per the previously mentioned convention, e.g., *Salmonella subsp. enterica* serovar Enteritidis being referred to as, simply, *S. Enteritidis*.

1.2. Physiology and Structure

Salmonella spp. are gram-negative, rods of varying dimensions (4). An important part of *Salmonella* structure is its multitude of surface antigens, most importantly the heat-stable lipopolysaccharide (LPS) endotoxins known as “O-antigens”, which are present in the outer cell wall membrane and important functions in the pathogenesis of *Salmonella* infections (4,13). These antigens are also the cornerstone of classic *Salmonella* serotyping, which groups

different variants depending on the type of the O-antigen (11,14). *Salmonellae* also possess – with some exceptions, as *S. Gallinarum* is always nonflagellate, as are infrequent strains of other serotypes – flagellar “H-antigens”, which are made of heat-sensitive, flagellin proteins in the tail like flagellum which enables bacterial motility (15). These may remain the same during the bacterial life cycle (monophasic) or be present in two phases (biphasic) (14). So-called “typhoidal strains,” responsible for enteric fever in humans, also have a polysaccharide capsule called the “Vi-antigen,” an important virulence factor that helps the bacteria by shielding it from host immune cells (12). This antigen is absent from most of the human pathogenic *Salmonellae*, the “Non-Typhoidal *Salmonellae*” (NTS), with the exception of *S. Dublin* (14). Many of these surface antigens may be altered, acquired, or partially or completely lost due to adaptive evolution or from microbe to microbe via horizontal gene transfer (HGT), through transduction, transformation, and conjugation (4,16). Apart from surface antigens, HGT can affect many other virulence factors and may contribute not only to increased virulence but also to antimicrobial resistance in *Salmonellae* (16,17).

Salmonellae (though capable of free-living) are mainly intracellular organisms (12,18). Unlike some other microbes known for their resistance to environmental stresses, *Salmonellae* do not form spores, but they can conglomerate as sticky, protective biofilms, which enhance colony nutrient and water binding and offer protection from host immunity and environmental hazards, including chemicals such as disinfectants and antimicrobials (4,16,19).

Although *Salmonella* spp. grows ideally at around 37°C and close-to-neutral pH, they can survive in wide ranges of temperature, acidity, and moisture (4,16). *Salmonella* can grow at a minimum temperature of 5.2°C (20). With rising air temperature, bacterial growth increases until about 42-45°C when bacterial proteins denature (16,20,21). However, thanks to a myriad of molecular stress-response mechanisms, some *Salmonella* strains withstand exposure to even higher temperatures, depending on the environment (22,23). Osmolality and desiccation (‘drying out’) in particular have been shown to increase resistance to extremes in both temperature and pH in *Salmonella*, and low-moisture foods, as well as high-fat-content ones, seeming to offer protection to the foodborne bacteria (24). *Salmonella* is also well known to survive freezing and retain its growth and infectious abilities after thawing; as a result, *Salmonella* outbreaks caused by frozen products have been known to occur (4,25-28). A large part of *Salmonella* overcoming high- and low-temperature stresses involves stress-induced changes in gene expression leading to the production of heat- and cold-shock proteins, sometimes resulting in permanently higher tolerance to sublethal heat or cold (22,23). Similarly, *Salmonella* relies on a variety of acid resistance mechanisms (ARMs) to adapt to a wide range

of environmental pH, being able to grow at pH levels ranging from 3.7 to 9.5 (16,23). Hours-long survival at around pH 2.5 and the ability to momentarily withstand pH as low as 2 have been documented and are thought to be due to a variety of acid-tolerance- and acid-resistance mechanisms (16,23). Temperature and acid resistance in bacteria are highly interdependent and may have overlapping effects, termed cross-tolerance (21,23). Aea et al. (1962) showed such a connection between acid and cold tolerance, as far back as the 1960s, with their experiment showing that acidic juice was better tolerated by *Salmonella* when frozen (29). It has since been demonstrated by numerous studies that different stresses, like acidity and cold or heat and starvation, and acidity and osmolarity, are linked at the level of molecular tolerance and resistance mechanisms (22,23). Cross-protective responses to different stressors may also help *Salmonella* withstand host immune responses and may even affect the expression of certain pathogenic effector proteins, although a 2021 review by Guillén et al. in *Foods* concluded that while a benefit to infective capabilities from these mechanisms may exist in some *Salmonella* variants, depending on circumstances, the costs of stress-induced adaptations may often outweigh the benefits for the surviving microbes and lead to decreased virulence (23,30).

Salmonella must also adapt to changes in nutritional availability as the bacilli go through their infective journey from food to gut to different kinds of cells (18). Recent genomic and molecular analyses have illustrated that *Salmonella* metabolism is remarkably flexible, and highly specific to the environment, such as the type of host cell (18,31-34). Research has shown, that during its life cycle, *Salmonella* utilizes nearly all major metabolic pathways while exploiting its host to acquire necessary substrates (34). Depending on nutrient availability and needs either the usual processes of aerobic metabolism or their alternative metabolic pathways may become dominant (31). *Salmonellae* are also facultative anaerobes, able to, in the absence of oxygen, ferment a variety of carbohydrates, producing acid and (sometimes) hydrogen disulfide gas (H₂S) (4). The carbohydrate most preferred by *Salmonella* appears to be glucose, but most carbohydrates are readily used, and even citrate alone will suffice as a carbon source (4,31). A classical characteristic of *Salmonella* spp. is that *Salmonella* – despite being by all intents and purposes generalists – does not ferment lactose or sucrose (4). However, several lactose fermenting strains, rare as they may be, have been described (14,35). Besides efficacious catabolism, *Salmonella* also depends on many biosynthetic pathways to acquire substrates such as amino acids, which may be either synthesized from scratch, scavenged from the host cell, or extracted from host cell proteins (31). This metabolic adaptability is important not only for meeting the bacterial energy needs of bacterial replication but also for

counteracting host immune defenses by out-competing native gut flora and resisting oxidative attacks by immune cells (30,31).

1.3. Pathogenesis

Human diseases caused by *Salmonella* include "enteric fevers" (typhoid and paratyphoid fever) caused by eponymous variants of subsp. *enterica*, and *Salmonella* gastroenteritis, termed salmonellosis, is also this thesis's primary topic (13). Salmonellosis is caused by various NTS, which are practically always acquired from animal sources since only the two typhoidal strains are restricted to human reservoirs (2,4). The primary cause of human salmonellosis is *Salmonella subsp. enterica*, found in various warm-blooded animals (4). After being shed in animal feces, *Salmonella* can either end up in soil or water and end up on things like vegetables or, more commonly, directly contaminate products from the host animal, such as meat, milk, or eggs (37). The mean infective dose of *Salmonella* is approximately 10^5 - 10^8 bacilli, although this may vary depending on both the food and the serotype in question: in the case of high-fat products, like chocolate, just a few individual microbes may be enough to cause an infection, due to the high-fat content providing the bacteria protection against gastric acid, the lack which is a risk factor for *Salmonella* infection (4,16,24). Medications counteracting or lowering that acid barrier (proton pump inhibitors or PPIs) have been associated with some enteric infections, and according to Hafiz et al., the association is particularly strong regarding *Salmonella*, several outbreaks of which in Europe alone have been linked to PPIs (37,38-40).

When consumed in great enough numbers enough bacilli can invade the enterocytes of the gut lining, specifically M cells in Peyer's patches (13). The invasion, possibly together with *Salmonella* enterotoxin, is thought to trigger the characteristic inflammatory response, leading to diarrhea (16,41). Murine models involving *S. Typhimurium* indicate that inflammation aids infection by inducing local changes in nutrient availability, providing the metabolically flexible *Salmonella* a competitive edge against the host's commensal gut flora, which are an important barrier against enteric infections (4,42,43). Some animal studies have shown increased levels – albeit only transiently – of *Salmonella* colonization following oral treatment with streptomycin, a broad-spectrum antibiotic, known to be detrimental to gut microbiota (44,45). Despite being able to replicate in the M cells, *Salmonella* tends to use them as a waypoint on their way to the underlying macrophages, which engulf the bacteria (17). *Salmonella* then proceeds to sequester itself within a modified vacuole, wherein it remains, replicating, until the

host cell undergoes apoptosis and releases the bacteria into the surroundings, restarting the cycle (16,41).

On average, *Salmonella* persists in the intestines until the infection is cleared by T and B cells, which can take anywhere from 6 to 12 weeks (46). The exact duration of bacterial shedding may be affected by antimicrobials, *Salmonella* serotype, immunosuppression, and patient age, with younger children shedding more bacteria for longer (4,47). Asymptomatic colonization, defined by most as the excretion of NTS in stool after 12 months, is thought to be rare in humans (<1%) (13,47). Although *Salmonella* infection does not typically result in long-term immunity, observational studies on African children do show an inverse association between antibodies during early childhood and bacteremia or invasive-NTS (iNTS) later in life, hinting at a possible protective immune response (48,49). Post-infectious complications, such as reactive arthritis, abscesses, or osteomyelitis, are rare in self-limited disease but complications may occur in as many as 10% of cases (13,57). A small number of animal studies suggest that NTS may prefer to concentrate and persist in lymphoid tissues, much like the typhoidal strains do in the gallbladder (47). Knowledge of chronic carriage and the persistence of NTS infections is scant, but some studies have found that chronic persistence of 30 or more days may occur in as many as 2-3% of cases (47,48).

Despite being a major cause of bloodborne infections in Africa, systemic invasion is relatively rare – though still severe – in the Western world (52-54). Bacteremia associated with *Salmonella* gastroenteritis, occurs in only 2-4% of cases, though children or the immunocompromised may be more susceptible to invasive disease (4,52,53,54). Increased rates of bacteremia have been associated with infections with certain serotypes, namely *S. Dublin*, which may be due to *S. Dublin* expressing certain virulence factors (55,56). The most common of the NTS, *S. Enteritidis*, has been considered weak in terms of invasiveness, however, invasive *Salmonella* infections have emerged as a major problem in the developing world, often caused by serotype *S. Enteritidis* (55). *S. Typhimurium* – specifically sequence type (ST) 313 – is another major cause of iNTS in Africa, and harbors high levels of resistance to multiple antibiotics (52-55,57).

1.3 Clinical signs

Typically, salmonellosis presents approximately 12 to 36 hours after consuming an infective dose of bacteria, although the incubation time ranges differ between sources (4,16,58). Although circumstances of infection and the serotype responsible may influence the clinical

picture, a typical presentation consists of diarrhea, abdominal pain, nausea, and vomiting (12). Low-grade fever or headache may also be present (4). Salmonellosis is usually self-limited in otherwise healthy persons and seldom lasts longer than a week (58,59). Some patients may require supportive treatment at a hospital, due to dehydration (12). *Salmonella* septicemia is usually not associated with gastrointestinal symptoms (4).

Due to the less typical but more severe clinical presentation, invasive NTS disease poses a diagnostic problem, especially in low-resource settings (53). In these cases, which are often due to HIV, non-specific symptoms form a clinical picture easily confused with other febrile illnesses (52,54).

Co-infections with other foodborne organisms, like *Staphylococcus aureus*, can also contribute to the clinical picture, as may have been the case in a *Salmonella* outbreak in Greece in 2016 when persons affected showed particularly severe symptoms (40).

Antimicrobial therapy is usually not indicated in otherwise healthy patients with simple gastroenteritis but is vital in reducing mortality in systemic cases, which may progress to septic shock if untreated (4). Antimicrobial resistance (AMR) is a serious problem in treating such cases and antimicrobial-resistant NTS infections are associated with more serious health outcomes, such as higher rates of complications, hospitalization, and mortality (36,60). This kind of general association with poor outcomes has already been established in the case of extended-spectrum beta-lactamase (ESBL) secreting bacteria (61).

1.4. Epidemiology

According to WHO's "Global Estimates and Regional Comparisons of the Burden of Foodborne Disease of 2010" and "WHO estimated of the Global Burden of Foodborne Disease" from 2015, illnesses from NTS, both diarrheal and invasive salmonellosis, resulted in the most significant disease burden of all enteric FBDs (62). The number of yearly cases estimated by these reports was roughly 380 million cases due to NTS (2,62). In the 2017 GBD study, however, it was estimated that each year there were more than 9 million cases of NTS enterocolitis, 535 000 cases of iNTS in the world, resulting in up to 129,700 deaths from enterocolitis, and as many or more for iNTS (52,62,63). The WHO's Foodborne Disease Burden Epidemiology Reference Group (FERG) determined some years ago in a 2016 report on Southeast Asia that approximately 16 000 cases and over 150 000 deaths were caused by NTS, 3600 of which were children under five (64). In Africa, *Salmonella* is estimated to be the single most common cause of fatal FBD, responsible for 32 000 deaths – which is more than

half of all *Salmonella*-related deaths globally – each year (63). Invasive NTS is concentrated in Sub-Saharan Africa, particularly among children (62).

Salmonella is not exclusively a third-world disease, however; according to the European Center for Disease Control (ECDC) and the European Food Safety Agency (EFSA), salmonellosis is the second most commonly reported foodborne gastrointestinal infection and a major cause of foodborne outbreaks (FBOs) in Europe, responsible for up to 20% of confirmed outbreaks as of 2022 as well as an estimated economic burden of over 3 billion euros each year as per a 2014 ECDC estimation (65-69). According to the data from the European Surveillance System – TESSy – presented in the ECDC’s Surveillance Atlas of Infectious Diseases and EFSA’s One Health Annual Zoonosis report for 2022, there were approximately 65 208 total reported cases and 47.122 confirmed cases (EFSA) of salmonellosis in the EU area over the year 2022 (65,69,70). The pooled temporal distribution of these cases, as seen in an ECDC figure (Figure 1), shows that in Europe salmonellosis tends to favor warmer months, peaking in early fall, whereas Figure 2 demonstrates that the most common age group affected in Europe is young children (69-70).

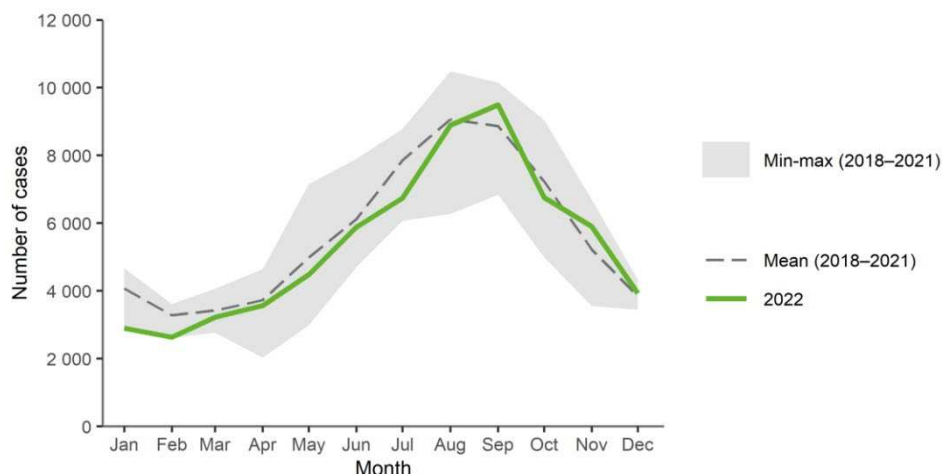


Figure 1. Confirmed *Salmonella* Cases by month in the EU/EEA (26 countries) in 2022 and 2018-2021.

Source: European Centre for Disease Prevention and Control (ECDC). Salmonellosis Annual Epidemiological Report for 2022. Stockholm (SE): ECDC; 2024. p. 4.

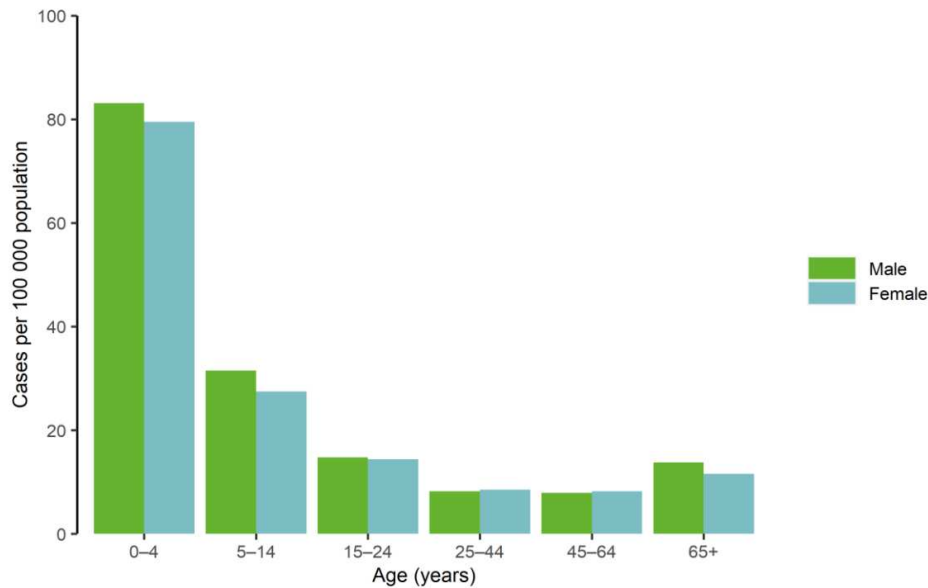


Figure 2. Confirmed salmonellosis cases per 100 000 population, by age and gender, EU/EEA (30 countries), 2022.

Source: European Centre for Disease Prevention and Control (ECDC). Salmonellosis Annual Epidemiological Report for 2022. Stockholm (SE): ECDC; 2024. p. 5.

According to the ECDC data, even though the total number of reported cases of *Salmonella* infections has increased slightly both in the EU and in the wider European Economic Area (EU/EEA), the incidence rate of case notifications (N/100 000) – that is, the notification rate (NR) – actually decreased slightly from 16.77 to 15.55 in the EU, and roughly as much in EU/EEA. One reason for this may have been the significant growth in the European population up to and during 2022 (69,71). Regardless, according to EFSA and ECDC, salmonellosis rates in Europe did not significantly differ between 2021-2022, nor has a trend in either direction that has been observed since a decade-long declining trend ended in 2016 (70,72). However, salmonellosis epidemiology varies by country (65,69).

The European country with the highest number of reported cases in 2022 was France (N=11162), but Czechia (N=7563) has the highest NR (NR=71.90) (69). This has been the case for Czechia for at least a decade (69). Czechia also had the lowest proportion of hospitalized patients, whereas Cyprus (87.1%), Greece (83.1%), and Lithuania (79.1%) have all hospitalized cases at over twice the rate of Europe on average likely due to mostly these severe cases being notified (69,70).

Salmonella infections reported in both the US and the EU/EEA decreased significantly between 2019 and 2020, during the COVID-19 pandemic (73,74). The number of deaths in the EU/EEA from salmonellosis decreased also between 2019 (N=93) and 2020 (N=61). The

European case fatality rate (CFR), the number of fatalities per 100,000 people, on the other hand, has not changed since 2019 and remains at 0.2 as of 2022 (69). Both infections and deaths have since seen slight increases as of 2021 in Europe but are still lower than before the COVID-19 pandemic (69,75). In the US, the pandemic did not appear to significantly affect the serotype distribution of human infection, but some changes have been observed in certain European countries, likely due to travel-related infections decreasing (73,75,76).

Overall, *S. Enteritidis* has been for years and remains by far the most common serotype found in European isolates from human salmonellosis patients, accounting for nearly half (48.9%) of all *Salmonella* infections reported in the EU/EEA in 2022 (70). The source of *S. Enteritidis* is most often eggs, which EFSA estimated in 2011 to be the source of 65% of all human salmonellosis (65,77). The second most common variant in Europe is *S. Typhimurium*, causing approximately 10% salmonellosis – slightly more than the monophasic variant, *S. Typhimurium* (1,4,[5],12:i-) (70). Rarer but still relatively common serotypes include *S. Infantis*, another serotype found in domesticated fowl. It is the causative serotype in ~2.3 % of European human NTS isolates (66,70). *S. Derby* and *S. Newport* each comprised around 1% of human isolates as of 2022 making them the fourth and fifth most common serotypes as of 2022 (65). Some data seem to indicate that the rare *Salmonella* variant, *S. Mikawasima*, may be becoming more prevalent in Europe (78). The number of *S. Mikawasima* infections has nearly doubled in Europe since 2009, and in 2013 the ECDC was alerted to an unusual increase in *S. Mikawasima* infections in humans (69,78,79). Since then, there have been several outbreaks of *S. Mikawasima* in Europe and, particularly in 2019, with the number of reports well over doubling between 2017-2019. (40,70,80-85). Some of the strains involved in several of these *S. Mikawasima* outbreaks (as well as some sporadic cases) have had troubling patterns of antimicrobial resistance, including a recent hospital outbreak of ESBL-positive *S. Mikawasima* in Split-Dalmatia County, Croatia (39,83-85).

Although most *Salmonella* infections are sporadic NTS are also responsible for most persons in the EU falling ill (N=6336) due to foodborne outbreaks (FBOs) (2,89). Over a thousand (N=1014) reported *Salmonella*-related FBOs in Europe in 2022; this makes *Salmonella* the second most common causative agent of all FBOs in Europe as of 2022, accounting for 17,6% of all reported FBOs (N=5871) in the EU/EEA area (65,70). Although this proportion is slightly less than that of 2021 (17.9%), the absolute number of FBOs-related cases reached its highest value since the early 2010s, in 2022 (72,86). This follows a decade-long trend in reported *Salmonella* outbreaks, which some studies estimated have been increasing by approximately 5% each year between 2015 and 2019, which may reflect changes

in surveillance (87). Importantly, as of 2022, NTS, together with *Listeria monocytogenes*, were also responsible for the majority (56.25%) of all human deaths linked to FBOs in the EU/EEA, with mortality more than doubling during the previous year (65,86). Although *Salmonella* accounted only for about 12.5% of these deaths, NTS alone was the cause of over half (50.5%) of FBO-related hospitalizations (65,86,87).

Perhaps unsurprisingly the most common causative serotype in *Salmonella* outbreaks is *S. Enteritidis*, though *S. Typhimurium* and its monophasic variant have also been involved in a large proportion of outbreaks in recent years (66).

The major sources, that have been identified, of *Salmonella* outbreaks in Europe in the past few years include eggs, "mixed (ingredient) food", meats (broiler and pig), and a variety of unspecified or processed products (86). During the 2018-2022 period, eggs were responsible not only for most *Salmonella* FBOs, but also for most human cases related to these outbreaks (66). However, the proportion of *Salmonella* vehicles related to FBOs varies somewhat year-by-year, and by country (66). Interestingly, in 2021, eggs – and all other foods – were surpassed by "vegetables and vegetable products," which is typically a relatively low-ranking vehicle for foodborne salmonellosis (66,88). The explanation for this probably lies in the largest *Salmonella* outbreak of that year, attributed to frozen tomato cubes served in Finland (28,88).

According to EFSA's Foodborne Diseases Atlas, 36 foodborne multi-country outbreaks (MCOs) affecting 18 countries in the EU/EEA happened in 2022 (65). 30, or over 83%, were caused by *Salmonella* (65). EU's Alert and Cooperation Network (ACN) and Rapid Alert System for Food and Feed (RASFF) received notifications about 7 of these 36 MCOs, although pose a significant human health risk, most of which likely involved *Salmonella* (86). Both resulting Rapid Outbreak Assessments (ROAs) by ECDC and EFSA (and one joint notification summary, a closed inter-agency document, regarding *Salmonella* Ball ST3502) in 2022 also involved *Salmonella* (88,89). ROAs are open-access glossaries of available information about cross-national outbreaks prepared by public health authorities when a human food or animal feed-related health risk, noted by European food safety systems, is considered particularly worthy of attention (89). Perhaps the most notable of ROAs from recent years was a large international outbreak of *S. Enteritidis* phage type 8, MLVA type 2-9-7-3-2, and 2-9-6-3-2, linked to eggs from Poland, in 2016 (90-94). This outbreak spread to an all-time high of 18 countries and led to 385 confirmed, and over 400 suspected human cases (90-94). This outbreak may have even affected the epidemiological data on serotype prevalence and distribution in affected countries in the surrounding years (75,92-95). In 2022, the ECDC reported 24 events of multi-country FBOs attributed to *Salmonella*, two of which received ROAs (70). The largest

and most threatening MCO seen as of late is the outbreak of monophasic *Salmonella* Typhimurium sequence ST34, first noted at the start of 2022 in the UK (96). Ultimately, the (two distinct) causative strains were linked to chocolate products manufactured in Belgium and sourced back to Italian buttermilk at the chocolate factories in question. The outbreak quickly led to 399 confirmed cases across 13 EU/EEA countries as of mid-2022, a large proportion of whom were children (10>years of age) and had a relatively high hospitalization rate (41%) (96). What makes this outbreak particularly threatening, apart from the average age and high HR of the persons affected, is that both bacterial strains involved were multi-drug-resistant (96-98).

1.5. Antimicrobial resistance

Antimicrobial resistance is a significant public health threat affecting all parts of the world. According to the WHO, the health burden of AMR far surpasses that of major public threats like tuberculosis and HIV, with tens of thousands of antimicrobial-resistant infections and subsequent deaths occurring in Europe alone (99). Regarding salmonellosis, the concern is the spread of bacterial resistance against antibiotics commonly used for treating severe or invasive infections and the resulting costs in morbidity and mortality, which are higher, at least in some ESBL-producing pathogens (61). Of particular note are fluoroquinolones, 3rd-generation cephalosporins, and macrolides, collectively termed "highest-priority critically important antimicrobials" (hpCIAs), as well as some other antimicrobials like tigecycline which are also sometimes used to treat multi-drug resistant *Salmonella* (99,100). Resistance to these common and invaluable drugs has already, at least to a degree, been observed in several pathogenic *Salmonella* variants in nearly all parts of the world (54). The mechanisms involved include a wide range of adaptations, including target enzyme mutations, efflux mechanisms, and antimicrobial degrading enzymes; unfortunately, many of these can be transferred from microbe to microbe through HGT, which may contribute to AMR against many important antimicrobials (101-103). For instance, *Salmonella* may acquire plasmid-related quinolone resistance (PMQR) genes horizontally, possibly contributing to AMR against the critically important first-line agent ciprofloxacin (103,104).

The food industry plays an important role in the emergence of resistant strains of *Salmonella* spp., as it does for other foodborne pathogens (105). The consumption of and resistance to several antimicrobials used in animals and humans are highly interconnected, albeit variable in strength and precise relation depending on the antibiotic agent (106). This is no surprise since, as discussed earlier in this text, *Salmonella* is highly adaptable when

repeatedly exposed to chemical hazards and can also acquire resistance genes through HGT from other strains or even genera of microbes, such as the gut flora of animals treated with antibiotics, a proven reservoir for resistance genes (16,107). The European health authorities' first joint report on AMR in 2015 determined that more antimicrobials were used in animals than humans in the EU/EEA; by the third report in 2022, the situation had nearly entirely reversed (106,108). This change has likely been largely due to increased vigilance and cooperation in surveillance and regulation as a part of the WHO One Health approach in humans, animals, and food. Still, overprescription and misuse of antibiotics remain an issue in the veterinary and medical fields (16,109).

Strategies, such as One Health, appear to have been relatively successful in areas like the US and Europe, with both seeing some decreasing resistance levels among many important human pathogens as of 2021 (69,110). However, in these areas, as well as in other parts of the world with similar trends, several strains still seem to exhibit troubling amounts of resistance to important antimicrobial agents (109,111). Despite clear decreases in antimicrobial resistance against most drugs in the US, the proportion of (animal) isolates resistant to critical – sometimes multiple – antibiotics has been on the rise among a set of specific NTS variants (110,112). Similarly, in an 8-year-long surveillance study on FBD-causing organisms and AMR in China, from 2023, *Salmonella* AMR levels varied considerably by both serotype and antimicrobial agent in question (113).

The European situation regarding antimicrobial resistance is examined annually in the ECDC's Annual Epidemic and Antimicrobial Resistance reports and EFSA's One Health Zoonoses reports, which, despite often only representing a fraction of confirmed cases, show much variability in resistance patterns depending on serotype, antimicrobial, and country (70, 88). Between 2013 and 2021, resistance to most antimicrobials seems to have decreased rather than increased for most serotypes, in most countries (65). However, similarly to the rest of the world, some serotypes show much higher resistance levels than others, and the same has been noted, for some of these variants, in the US as well (110). *S. Kentucky* seems significantly overrepresented among NTS with decreased susceptibility (114,115). In Europe *Salmonella* remains relatively susceptible to the most important antimicrobials, though resistance rates among *Salmonella* spp. for ampicillin (amoxicillin) and tetracyclines remain “high” as per EFSA's definition (>20%-50% of tested isolates resistant) and that of cephalosporins “moderate” (>10-20% of tested isolates) (Figure 3) (115). All three aforementioned classes of antimicrobials, though, have in recent years shown decreasing trends in more countries than the opposite (115). As of 2022 European *Salmonella* spp. isolates from humans are "only"

moderately (18.7%) resistant to ciprofloxacin, and only a little more than 1% exhibit what is termed “high-level resistance” (115). Among the most common human pathogenic serotype, *S. Enteritidis*, EU/EEA data show slightly higher levels ciprofloxacin resistance (22.6%), whereas among *S. Infantis* isolates the proportion is as much higher (115). In the 2022 EFSA report, an increasing trend in *S. Enteritidis*' resistance to ciprofloxacin was also noted to have been observed over the past decade in 10 countries, whereas only 5 countries showed a decreasing trend (16,115). Fortunately, the combined resistance to ciprofloxacin and cephalosporins has remained very low in human NTS (<1%) in the EU/EEA as well (115).

EU total	AMP		SMX		TET		CIP		CTX		Combined CIP/CTX	
	N	% res	N	% res	N	% res	N	% res	N	% res	N	% res
<i>Salmonella</i> spp. (27 MSs)	16,059	25.2	8596	25.6	14,305	25.1	15,824	18.7	15,323	1.4	15,264	0.9
<i>S. Enteritidis</i> (26 MSs)	4629	5.1	2637	3.1	3867	4.3	4431	22.8	4226	0.4	4191	0.2
<i>S. Typhimurium</i> (27 MSs)	1971	32.1	941	30.2	1633	26.8	1963	19.6	1839	1.0	1833	0.4
Monophasic <i>S. Typhimurium</i> (19 MSs)	2587	86.7	1673	78.5	2528	77.3	2588	9.6	2588	1.8	2587	1.0
<i>S. Infantis</i> (26 MSs)	705	14.9	305	50.5	656	38.1	701	40.1	698	6.0	698	5.9
<i>S. Kentucky</i> (18 MSs)	228	59.2	169	60.9	213	67.1	227	72.7	227	12.3	227	12.3

Figure 3. Occurrence of resistance to selected and critically important antimicrobials in *Salmonella* spp. serovars in humans, 2022 (in the EU/EEA).

* %res = percentage of resistance; AMP = ampicillin, CIP = ciprofloxacin/pefloxacin, CTX = cefotaxime, N = number of *Salmonella* isolates tested; SMX = sulfamethoxazole, TET = tetracycline.

Source: European Food Safety Authority (EFSA). The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2021-2022. EFSA J. 2024 Feb 1;22(2): e8583. p. 16.

Multi-drug resistance (MDR), as defined by EFSA as simultaneous resistance against three or more antimicrobial classes, remains an issue in Europe: A monophasic variant of *S. Typhimurium*, specifically of the antigenic formula 1,4,[5],12:i:-, has for years shown very high levels of MDR, although a small decrease did occur between 2019 and 2022, with MDR prevalence among human isolates of the variant dropping from nearly three fourths to 68.2% (114,115). MDR, in general, across all *Salmonella* spp. in the EU/EEA, seems to have remained at high- (~23%) but relatively stable levels in both human- and animal isolates between 2020-2022 (114,116).

ESBL-producing *Salmonella*, with resistance to common beta-lactam agents like penicillins and 3rd generation cephalosporins, also appear stable in the UE/EEA: Although the amount of presumptive ESBL-producing isolates increased slightly (0. 2% increase) between 2019 and 2021, the range (0.1 or 0.2-3.5%) of reported proportions in *Salmonella* spp. isolates between countries have stayed at either very low or low levels in both humans and animals

(114). Isolates expressing specific Amp C cephalosporinase-enzymes were also rare (114). *S. Infantis* and *S. Kenya* were the serotypes most often found to be ESBL positive at 5.2% and isolates at 5.3%, respectively. *S. Kentucky* was also relatively well represented among ESBL *Salmonella*, and in 2017, these ESBL *S. Kentucky* strains were found to carry carbapenemase genes (116).

Carbapenem-resistant Enterobacteriaceae (CRE), due to the production of carbapenemase-enzymes, are even higher-priority micro-organisms to look out for, as carbapenemases are often considered “last resort” antimicrobials (115). In 2021-2022 data EFSA four *Salmonella* spp. isolates were found to be positive for carbapenemases (115). Several non-typhoidal *Salmonella* serotypes, like *S. Infantis*, *S. Kentucky*, *S. Typhimurium*, and *S. Saintpaul*, possessing carbapenem resistance have been reported in both humans and animals in nearly all parts of the world, including Europe in the past decades (117,118). Luckily, though, these carbapenemase-positive NTS are very rare, and between 2018-2021, no such *Salmonella* isolates were found in Europe (although testing for meropenem resistance was not very sensitive in earlier years of this period) (99,115,117).

1.6. *Salmonella* in Croatia

Salmonella epidemiology in Croatia largely lines up with Europe in general. Since at least 2005, salmonellosis has been the number one zoonotic disease and the most reported food-borne infection, making up 19.2% of the latter as of 2021 (66,86,119). The number of confirmed human salmonellosis cases was approximately 1047 in 2022, hundreds fewer than in the pre-pandemic year of 2019 but almost 100% more than in the previous year of 2021 (69). Interestingly, unlike in most of Europe, the total number of salmonellosis cases declined between 2020-2021, being at its lowest (N=593) during the decade, after 2013 during which the lowest number (N=592) of cases were recorded in Croatia (69,119,120).

Despite the number of cases nearly doubling between 2021 and 2022, with 1047 confirmed cases in 2022, the overall trend seen in ECDC data appears to have been a declining one pre-pandemic, an observation noted as far back as 2017 in The Croatian Food Agency (HAH) Zoonoses report for 2015-2016 (69,119). However, in 2022, the notification rate (N/100 000) in Croatia was the 5th highest (NR=27.11) in the EU/EEA area for salmonellosis (69). Most of these cases were domestic rather than travel-associated (69). Some older sources from the past decade name eggs as the most common source of foodborne *Salmonella* infections in Croatia, at least in some parts of the country, with cakes and baked goods prepared with eggs

(121,122). EFSA data on foodborne outbreaks also indicate that miscellaneous and mixed-ingredient foods, such as cakes or biscuits, have been the most common vehicles for *Salmonella* outbreaks in Croatia since at least 2018 (86).

S. Enteritidis, like elsewhere in Europe, appears to be the most common serotype found in Croatia, with some sources from the past 10 years suggesting *S. Enteritidis* comprise 80 to 90% of all Croatian isolates (69,121-123). In the ECDC data, even when accounting for serotype data completeness (87.9%), the proportion of reported *S. Enteritidis* cases is somewhat smaller, with 345 cases reported (69). *S. Typhimurium* is another common variant in Croatia, and in the ECDC data, cases related to it appear to be increasing in numbers as of 2022 (69, 122,123). Evident also in the ECDC data, Croatia has had relatively high amounts of the relatively rare (NR=0.1) serotype *S. Mikawasima*, which is only reported at a higher rates in Denmark and Norway (NR=0.27, NR= 0.17), wherein this variant has seen more stable rates over the years (69). A recent three-year-long surveillance study by Carev et al., examining *Salmonella* spp. in Split-Dalmatia County (SDC) in 2020-2022, found that among both reported hospitalized patients and outpatients in SDC, *S. Mikawasima* has surpassed other serotypes, and is, as of 2022, the most common serotype in the county (84).

Outbreaks of salmonellosis are reported in Croatia when at least two people are infected from the same source (88,123). In the EFSA foodborne outbreak surveillance data from 2022, Croatia had only one confirmed (“strong evidence”) *Salmonella* outbreak, out of eight total confirmed food-borne outbreaks in 2022 (69). Outbreaks of salmonellosis regardless of strength of evidence accounted for 9 out of 27 FOBs during 2022, and among these were 83 affected persons, six of whom were hospitalized (69). Both affected persons and hospitalization rates mirror those from 2020 and 2021, only differing by a few cases, meaning the impact of 2022 foodborne *Salmonellosis* outbreaks in Croatia has remained significantly below pre-COVID-19-pandemic levels (69). No deaths due to *Salmonella* outbreaks in Croatia have been reported in European surveillance data from 2018 to 2022 (69,86). The last known fatal case occurred in 2016 as a part of the multi-country outbreak of *S. Enteritidis* linked to eggs from Poland, which had four confirmed cases in Croatia, one of which – that of a 5-year-old patient–proved fatal (124). As mentioned previously, mixed or composite foods are the most common foods responsible for outbreaks as of 2022, followed by meats, meat products, and eggs (86). Outbreaks linked to more unusual sources have also occurred, such as one that occurred in Šibenik-Knin county, in the 50 0000 inhabitant town of Šibenik in 2014, wherein untreated drinking water from a local spring contaminated with *S. Enteritidis* was connected with several cases of gastroenteritis (125).

The age-standardized salmonellosis rates, according to ECDC, unsurprisingly enough, show that the age groups most commonly affected in Croatia are the very young, with 0-5-year-olds particularly overrepresented (69).

The Antibiotic Resistance in Croatia, 2022 summary results (as seen in Figure 4) by The Croatian Intersectoral Coordinating Mechanisms (ISKRA) and the Croatian Academy of Medical Sciences showed that the antimicrobial with the highest level of resistance against among *Salmonella* spp. was ciprofloxacin (18% resistance among tested isolates) (126). This was followed by ampicillin (amoxicillin) at 16% of isolates showing resistance to the agent. The lowest resistance levels were found with ceftriaxone and ceftazidime, at 2 % each (126).

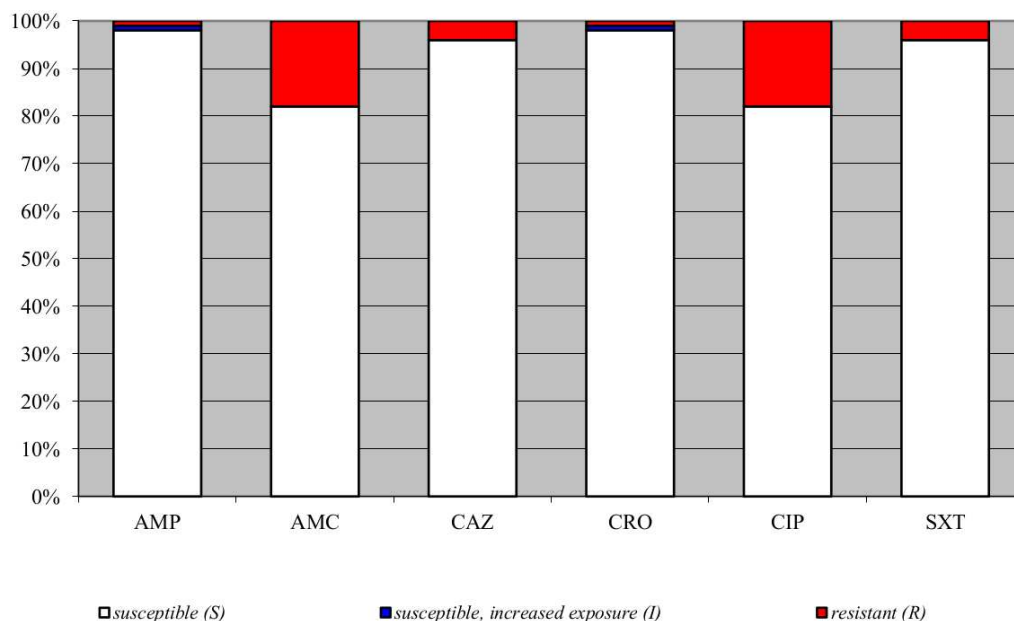


Figure 4. Antimicrobial resistance for 2022 in *Salmonella* spp., summary results for the isolates from 39 centers in Croatia.

*AMP = ampicillin, AMC= amoxicillin-clavulanate, CAZ = ceftazidime, CRO= ceftriaxone, CIP= ciprofloxacin, SXT= trimethoprim-sulfamethoxazole

Source: Tambić Andrašević A, Žmak L, Obrovac M, Hunjak B, Babić-Erceg A, Unukić T et al. Izvještaj o osjetljivosti i rezistenciji bakterija na antibiotike u Republici Hrvatskoj u 2022.g. Croatian Academy of Medical Sciences public health collegium committee for antibiotic resistance surveillance in Croatia. Zagreb (HR): The Croatian Academy of Medical Sciences; 2023. Report No.: 1846-1654. [Croatian, English]. p. 73.

There has been a large increase in ciprofloxacin resistance among *Salmonella* spp. in Croatia as well: in 2013 all isolates were susceptible to ciprofloxacin and resistance to nalidixic acid, an indicator of resistance to quinolones, was as low as 2% (126). The steepness of the increase postulated by the report may have to do with Europe-wide changes in AMR-testing indicator antibiotic to a more accurate one in 2014, and the low numbers of reported cases during the COVID-19 pandemic, but the report concludes that a true increasing trend in

ciprofloxacin resistance does exist in Croatia, mirroring the global trend (126). Luckily, ESBL isolates have remained uncommon in Croatia, and the proportion of resistant strains appears to have stayed stable (126).

In a 2006 paper, Petanović et al. analyzed the trends in resistance to antibiotics of various pathogens in Slavonski Brod in 2005 compared to 2004 (127). They found that *Salmonella* spp. resistance to ampicillin was much higher in Slavonski Brod (22%) than in the rest of Croatia at the time (6%), possibly suggesting some regional variability within Croatia regarding AMR among *Salmonella* spp., at least at the time of the paper's publishing (127). More recently, Carev et al. found that resistance levels against ceftriaxone and ceftazidime were somewhat higher than in the country as a whole, whereas ciprofloxacin resistance was less common (84). In addition, the study in question identified 31 cases of ESBL positive *Salmonella*, namely *S. Mikawasima*, during this time, some of which possibly in connection with a hospital outbreak at a neonatology ward in Split during the COVID-19 pandemic (83,84).

1.7. Diagnostic Methods

Diagnostic methods for *Salmonella* have classically relied on culturing from bodily fluid, usually fecal samples, biochemical tests, and surface antigen agglutination tests (11,14). These isolation, identification, and serotyping methods are well-established and affordable, and remain the gold standard even into the 2020s (128,129).

Under ideal conditions, positive *Salmonella* culture is both specific and selective, although in practice factors like individual differences in fecal shedding, long transport, suboptimal storage, and antimicrobial treatment can each affect culture results (14,128,129). In addition, the acquisition of pure culture usually involves incubation, followed by re-plating and re-incubation, taking altogether at least 48 hours and making this method a rather time-consuming one (128,129). Due to samples often containing other – highly fastidious – enteric pathogens, culturing of *Salmonella* requires differential media such as Salmonella-Shigella agar and Selenite broth, although others such as 5% sheep's blood agar, Mueller-Hinton agar, MacConkey agar, Hektoen Enteric (HE) agar or Xylose-Lysine-Deoxycholate (XLD) may be used (14). *Salmonella* colonies' morphology alone is inadequate for identification, however, so confirmation of bacterial species is done on *Salmonella* cultures, using biochemical and serological tests is often done (128). Biochemical identification has classically involved the triple sugar test on Kligler's Iron-agar (KIA), which is based on different carbohydrate

fermentation patterns exhibited by different bacteria (14). Fermentation of either lactose, sucrose, or both, to acid-induced color changes on KIA and possibly observable gas bubbles, and sometimes black staining from H₂S gas (14). As of recent, more comprehensive biochemical identification systems have become commonplace, with a working principle similar to the triple-sugar test: color indicators for different chemical reactions are used to reveal the biochemical properties of the pure cultured bacteria (14). *Salmonella* subsp. enterica most often (90%) tests positive for fermentation of most tested carbohydrates, utilization of most amino acids, and conversion of nitrates to nitrites (14). They rarely (5%) or never produce indole, ferment lactose or erythritol, deaminate phenylalanine, hydrolyze urea, gelatin, or tyrosine, and are oxidase and lipase negative (14).

Serotype determination by tube or slide agglutination remains a common method for typing in many European reference laboratories (130,131). It relies on the detection of an agglutination reaction with antisera for different surface antigens (11). It is considered a specific, reliable technique, although, its drawbacks include need for a wide variety of reagents, skilled test conductor, and its interpretation depends on the skills of the person performing the test (11). O-antigen serotyping begins by first mixing pure colony with polyvalent sera to further confirm the species and *Salmonella* group with O-agglutinability. The sera are tested for starting with polyvalent O-antisera, followed by monovalent O-antisera for serogroup and type determination, which narrows down specific O-antigens to test for (14). H-antigens are tested for by a similar principle using H antisera but involve culturing on Sven Gard medium and repeating the agglutination test on the culture for determining phase inversion (e.g., is the strain mono- or biphasic) (14). The antigen agglutination results are then written down into antigenic formulae (7).

More sophisticated ways of identifying and serotyping *Salmonella* include immunological assays, optical or electrochemical methods, and molecular methods (11,132). The molecular methods, like multiple locus variable number tandem repeats analysis (MLVA) and whole-genome sequencing (WGS), have been deemed very advantageous in identifying and differentiating *Salmonella* strains and even for tracking antimicrobial resistance genes (36, 131,133). Both are based on nucleic acid hybridization and are highly sensitive, specific, and quicker than classical methods, but they are still limited by the skill, cost, and need for bacteria and even variant protocols (134,135). Seeing as they're able to differentiate particularly fast-evolving strains are especially suited for surveillance and epidemiological purposes, allowing easier and quicker “source attribution” for outbreaks (131,133). As of 2022 ECDC and the public health authorities in multiple European countries, as well as the CDC in the US, use

MLVA and WGS in both routine surveillance and outbreak investigations (133-135). Another method occasionally used for *Salmonella* strains is bacteriophage lysotyping, wherein a serotype is identified based on its characteristic susceptibility pattern to lysis by different kinds of bacteriophages (14,70).

Antimicrobial resistance testing, though direct detection of resistance genes is possible using methods like WGS, is still most commonly determined, in Europe, by the minimum inhibitory concentration (MIC) needed to halt observable growth of either broth- or agar plate cultures (36). This “disk diffusion test” involves culture-saline suspension cultivation on a Müller-Hinton agar plate with antimicrobial-impregnated paper disks, the visible colonies’ distance (zone of inhibition) from which is measured post-incubation (14). The zone of inhibition for each antimicrobial is compared with a standardized cut-off value, representing the MIC, and the tested isolate is deemed either susceptible, resistant, or intermediate; S, R, or I to the agent (136,137). The intermediate results have caused some confusion concerning the likelihood of treatment success with such agents, but the 2020 definitions from European Committee on Antimicrobial Susceptibility Testing (EUCAST), the body responsible for European cutoff values, emphasize that the susceptibility in such cases depends much on the exposure time and amount, with “I” often being closer to “S” given an increase in dose (137).

2. OBJECTIVES

The aim of this study is to examine the epidemiology and antimicrobial resistance patterns of non-typhoidal *Salmonella* infections in outpatients in Split-Dalmatia County in 2022.

Hypothesis: there exist patterns distinct from national and general European ones in regard to serotype prevalence and antimicrobial resistance among *Salmonella* spp. in Split-Dalmatia County.

3. MATERIALS AND METHODS

The data used for this study consisted of an anonymized computerized database of *Salmonella* isolates from outpatients from 2022, courtesy of the Teaching Institute of Public Health of Split-Dalmatia County. Data were used with ethical permission (no. 2181-103-01-24-24) from the Institute.

Laboratory methods for isolation and identification of *Salmonella* from patient stool samples included inoculation of Selenite broth with a stool sample, followed by 24h incubation at 37°C, for enrichment, then followed by plating on SS agar, from broth and further incubation at 37°C for another 24 hours. The resulting colonies, with the appearance suspicious of being *Salmonella* was then transferred into Kligler's Iron-agar (KIA) tubes and again incubated for 24h at 37°C. The KIA colonies, post-incubation, were screened for changes characteristic of *Salmonella*. In addition, a commercially available 24h biochemical test API 10 S (bioMérieux, Marcy l'Etoile, France) for identifying *Enterobacteriaceae* was used for identification based on carbohydrate and amino-acid reactions.

Salmonella serotyping was done using slide agglutination testing. *Salmonella* colonies were mixed with a drop of antisera on a slide, with saline control, and observed for up to 30s for an agglutination reaction; first, with, polyvalent *Salmonella* antisera followed by monovalent O and H *Salmonella* antisera.

Antimicrobial susceptibility testing was carried out according to the EUCAST recommendations, version 2022-01-01 using the disk diffusion method (136). Culture-saline suspension of 0.5 McFarland in density on Muller-Hinton agar, with 7 antimicrobial disks, was incubated for 24h at 37 C, followed by the measurement of the distance from the disk to the end point of no visible growth through the back of the dish, against a dark background. The distance from the disk to the end point of no visible growth constitutes a zone of inhibition. Interpretation was done using the EUCAST clinical breakpoints v.12.0 (Table 1.) (136). The antimicrobial agents examined included ampicillin, ceftriaxone, ceftazidime, amoxicillin-clavulanate, chloramphenicol, trimethoprim-sulfamethoxazole, and ciprofloxacin.

Table 1. EUCAST breakpoints for *Enterobacteria* and *Enterobacteriales*.

Antimicrobial	MIC			Disk content (mcg)	Zone of inhibition (mm)		
	S>	R<	ATU		S>25	R<	ATU
AP	8	8		10	14A*	14A	
CRO	1	2		30	25	22	
CAZ	1	4		10	22	19	
AUG	83	83		20-10	19A	19A	19-20
C	8	8		30	17	17	
TS	2	4		1.25-23.75	14	11	
CIP	0.25	0.5	0.5	5	24	12	

*A. Ignore growth that may appear as a thin inner zone on some batches of Mueller-Hinton agars.

† S = susceptible, R = resistant, ATU = Area of Technical Uncertainty

AP = ampicillin, CRO = ceftriaxone, CAZ = ceftazidime, AUG = amoxicillin-clavulanate, C=chloramphenicol, TS = trimethoprim-sulfamethoxazole, CIP = ciprofloxacin.

Source: European Committee on Antimicrobial Susceptibility Testing (EUCAST).

Breakpoint tables for interpretation of MICs and zone diameters, Version 12.0, valid from 2022-01-01. Växsjö (SE): EUCAST; 2022. p.15-19.

3.1. Data analysis

Basic statistical analysis, such as was performed using in-built statistics options in LibreOffice Calc-spreadsheet software version 7.3.7.2 (The Document Foundation, Berlin, Germany) (138). Map created using with QGIS-software ver. 3.34.3 (QGIS Association, Grüt, Switzerland) (139). County boundaries data were created from geoBoundaries project database of global administrative boundaries (geoLab, Williamsburg, Virginia (US)) (140).

The number of cases, incidence rate, and incidence rate were calculated based on a sample and 2021 census data obtained from the Croatian Bureau of Statistics (141). Further statistical testing was done with appropriate Chi-squared-tests available on the MedCalc online statistical calculator: One-way chi-squared, two-way chi-squared, and the “N-1”-chi-squared (for comparison of proportions), with a significance cutoff value of $P < 0.001$ (142-144).

Sex distribution, as well as other distributions within the study population, were tested with one-way Chi-squared with the null hypothesis assuming no difference. Additionally, a comparison of the distribution of parameters such as sex or age between the sample and Split-Dalmatia County (as per 2021 census records), as well comparison of parameters (e.g. sex, serotype) between intra-sample groups (e.g. age group, isolates grouped by month of isolation), were performed with a two-way chi-squared test. The chi-squared test for independence was

utilized when testing for a link between proportions (for parameters like age, sex, serotype, town, and month of isolation).

4. RESULTS

4.1. Epidemiology

The data set included 165 laboratory-confirmed salmonellosis cases in outpatients in Split-Dalmatia County (SCD) in 2022, while repeat infections (“copy strains”) in the same patients were excluded. Variables recorded with laboratory-confirmed salmonellosis cases included sex, age, place of residence, serotype of the isolate, and the month of isolation. Complete data on all aforementioned variables was available for only 81 patients or 49.1% of the sample.

Data on sex was available for the entire sample and is visualized in Figure 5 below. Males (N=80) made up 48,48% of the sample and females (N=85) 51,52%. No statistically significant ($\chi^2=0.152$, $P=0.697$) disparity in the sex distribution in salmonellosis outpatients in the Split-Dalmatia, with no significant difference between this and the total SDC population (as per 2021 census) extant for either males ($\chi^2=0.017$, $P=0.898$) or females ($\chi^2=0.017$, $P=0.898$).

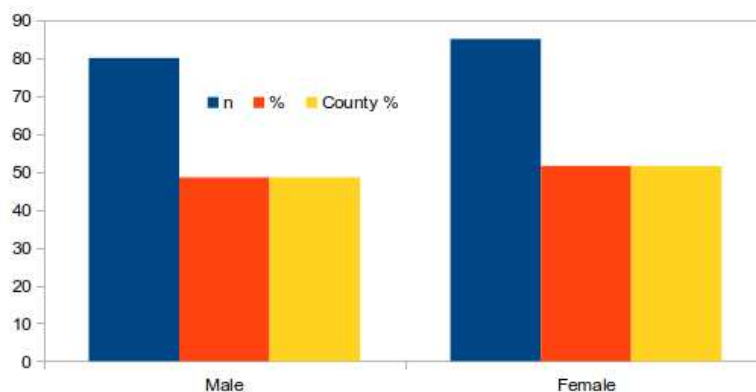


Figure 5. Sex distribution in outpatients with salmonellosis in the Split-Dalmatia County in 2022 and in County 2021 population.

*N=number of cases, %=proportion of male and female salmonellosis outpatients, County %=proportion of males and females in the Split-Dalmatia County in 2021 census data.

Age was recorded for 100, or 60.61% of the 165 salmonellosis outpatients. The age of outpatients in 2022 ranged from 0 to 88 years, with a mean age of 24.28 ± 28.39 . For the analysis, the data regarding age were categorized into 10 groups: 0-5; 6-9; 10-19; 20-29; 30-39; 40-49; 50-59; 60-69; 70-79; 80-88 and proportion of the total was calculated for each group (Figure 6). We see clear skewness (0.802) to the right, with a remarkably higher proportion of ages 0-5 years age group in comparison with other age groups. No statistically significant connection between age group patient sex was found ($\chi^2=11.824$, $P=0.223$).

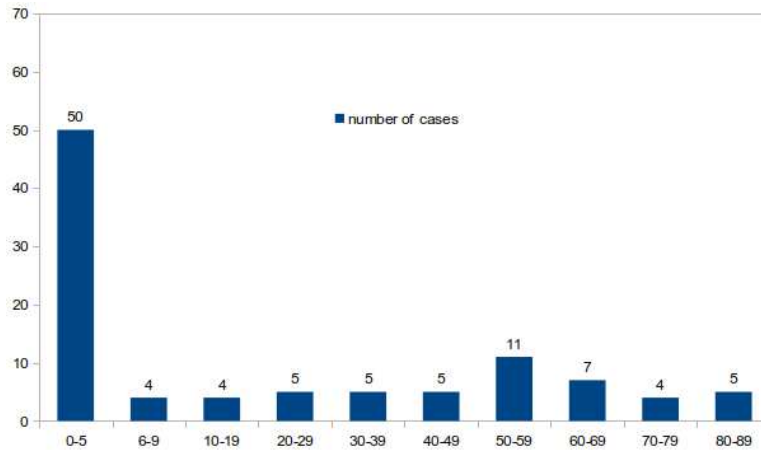


Figure 6. Age distribution of salmonellosis outpatients in Split-Dalmatia County (N=100)

City, town, or municipality (here-on referred to simply as “town”) of residence were recorded for 153 patients. The number of salmonellosis cases among outpatients in each SDC town is shown in Figure 7.

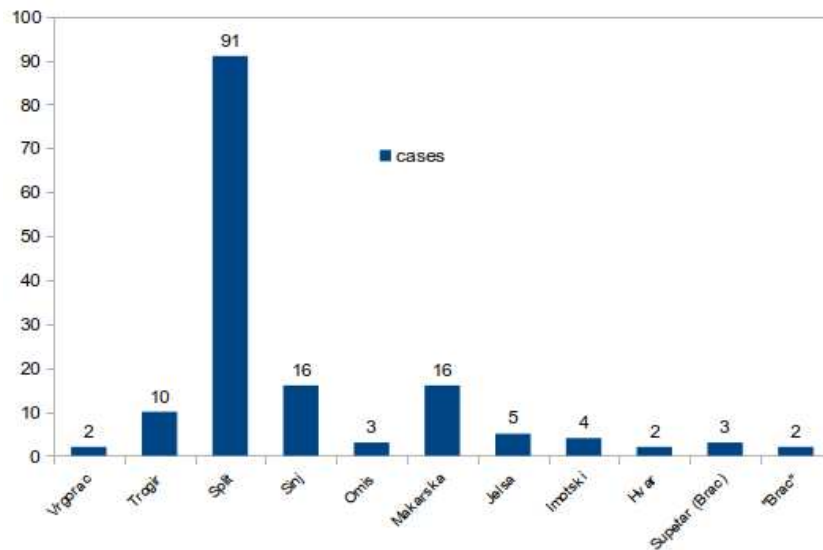


Figure 7. Number of reported out-patient salmonellosis cases in Split-Dalmatia County per town (N=153)

When accounting for population, the smaller towns, Makarska and Jelsa, have a higher number of cases per 1000 population than larger towns like Split (Figure 7, Figure 8). Interestingly, Jelsa, the smallest of the towns, has the highest number of cases concerning its population in the County. On the other end, Vrgorac had only two cases, as did Brač and Hvar, but a larger population, meaning it also had the lowest incidence. It does bear mentioning, though, that for some patients the town is reported as Supetar, which is the largest settlement on the island of Brač, whereas for others “Brač”, without specifying the settlement, is recorded.

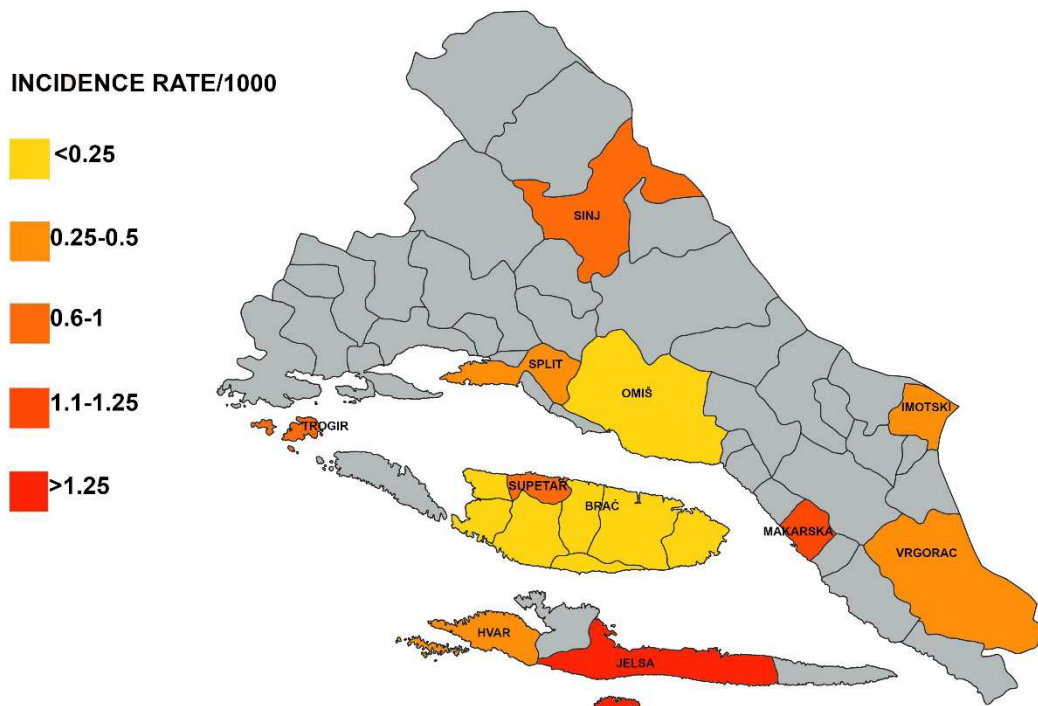


Figure 8. Salmonellosis incidence per 1000 person in the outpatient population of SDC by town. Darker shades correspond to higher incidence, lighter to lower.

Temporal distribution by month in 2022 (Figure 9) was examined using the month of isolation (1-12) for each case in the sample; data on this was available for 157 cases. The absolute highest number of cases (N=27) was recorded in September and August (N=20). Other peaks occurred in March, and December with 17 cases each. The lowest number of cases was recorded in November (N=5) and April (N=6). Examining age distribution per month was complicated by large amounts of missing data on patient age. However, as seen in Figure 10, the 0-5-age group dominates the most months, as it does the whole sample, with all but one case in March occurring in this age group.

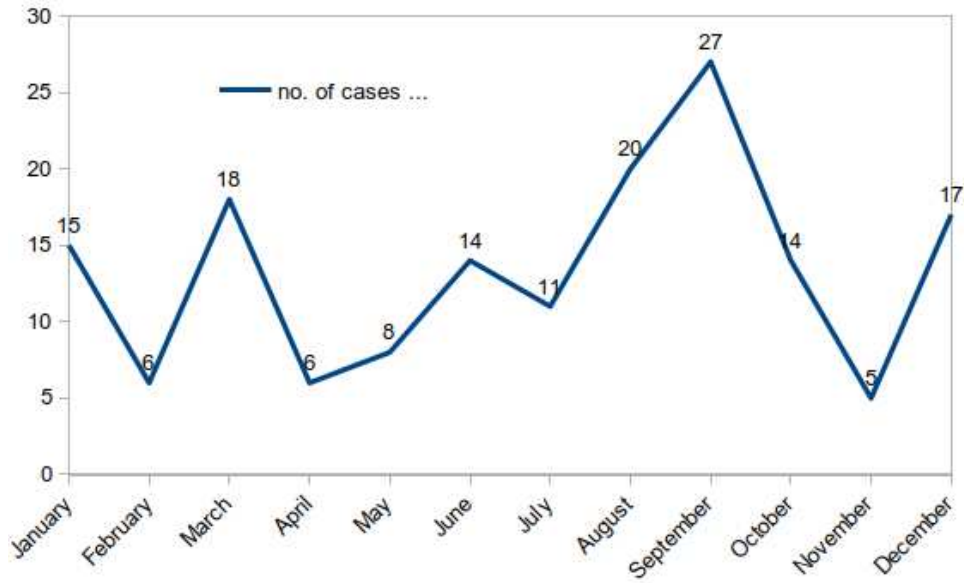


Figure 9. Salmonellosis cases in outpatient in SCD by month over 2022

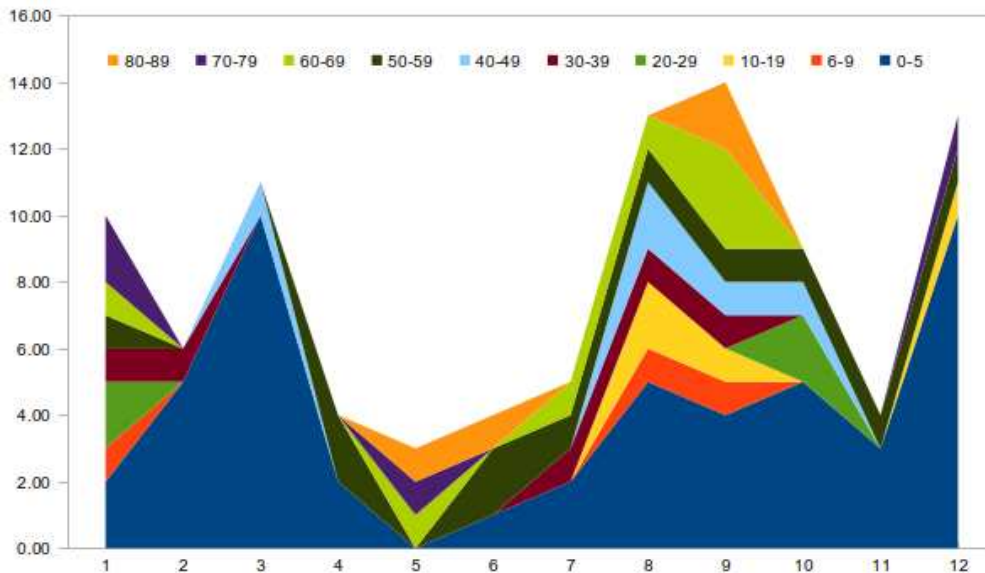


Figure 10. Age distribution of SDC outpatient salmonellosis cases by month.

Data on serotype was available for 149 patients, with the remaining 16 (9.697%) of the 165 isolates being recorded simply as *Salmonella* spp. The most commonly identified serotype was *S. Mikawasima* (N=35), with it making up more than 21% of all cases and approximately 24.5% of all identified serotypes in the study population (Figure 11). *S. Enteritidis* was the second most common serotype found in our sample (N=30, 18.182% of cases, 19.868% of recorded variants). *S. Typhimurium* (both phase-variants) was the third most common variant (N=17; 10.303%; 11.258%). Several rare variants only had one case recorded.

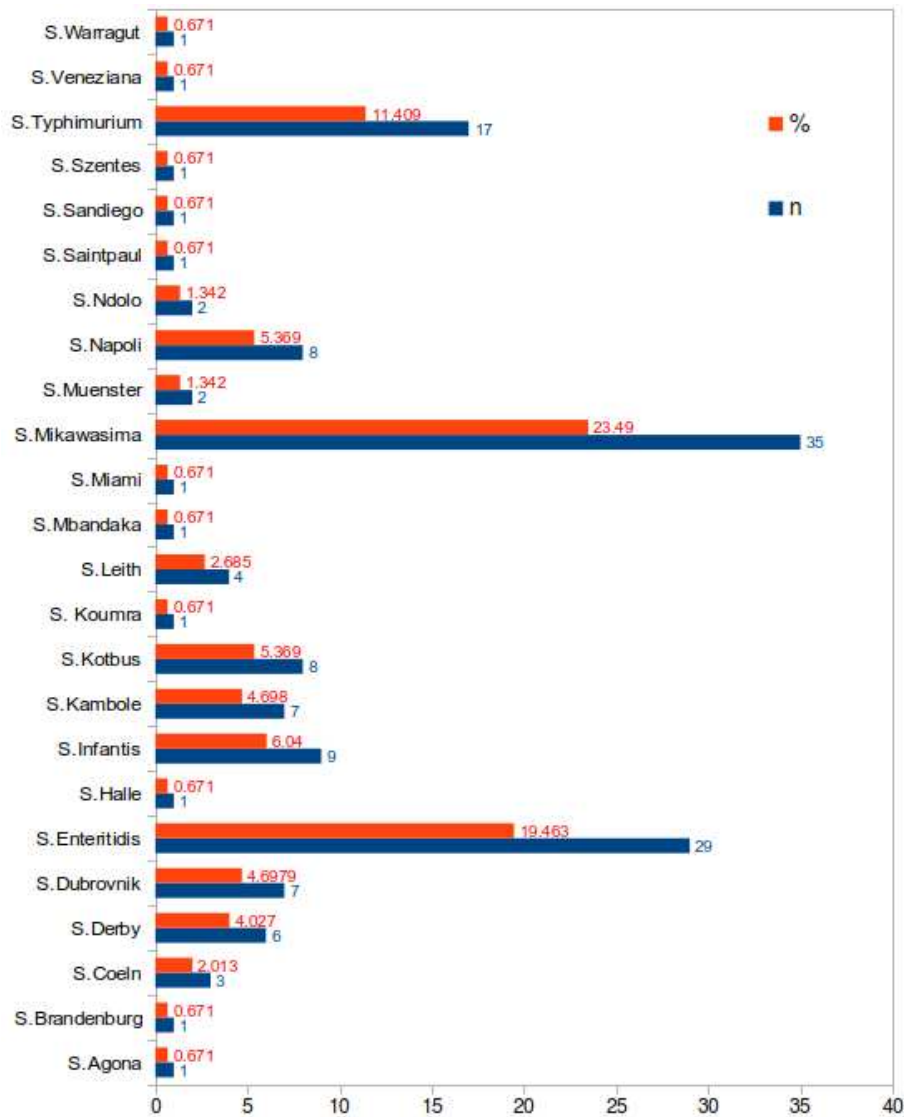


Figure 11. Number of isolates by serotype and their proportion (%) of *Salmonella* spp. isolates with known serotypes (N=149) in the outpatient population of Split-Dalmatia County.

Using chi-squared for each variant with three or more isolates (11 variants, 134 isolates) no statistically significant difference was found in variant occurrence between males and females in the study population ($P > 0.001$ for all variants).

Age distribution by serotype (Figure 12) on the other hand shows a statistically significant difference in serotypes in the 0-5 age group ($\chi^2 = 103.957$, $P < 0.0001$). In this age group, *S. Mikawasima* (N=23) makes up a notably large proportion (48.94%) of isolates (N=47) in that group. These cases in the 0-5 age group also making up well over half (65.71%) of all 35 *S. Mikawasima* cases. Data on age was available for the vast majority of *S. Mikawasima* isolates (N=29 or 82.86% of isolates of this serotype), but no age was recorded in 6 cases. In contrast to *S. Mikawasima*, the second most common variant, *S. Enteritidis* is

distributed relatively equally among age groups, with the highest number (N=4) of cases in the 0-5 age group and the highest proportion (75% of cases in the age group) among the oldest age group of 80-88 year-olds (N=4).

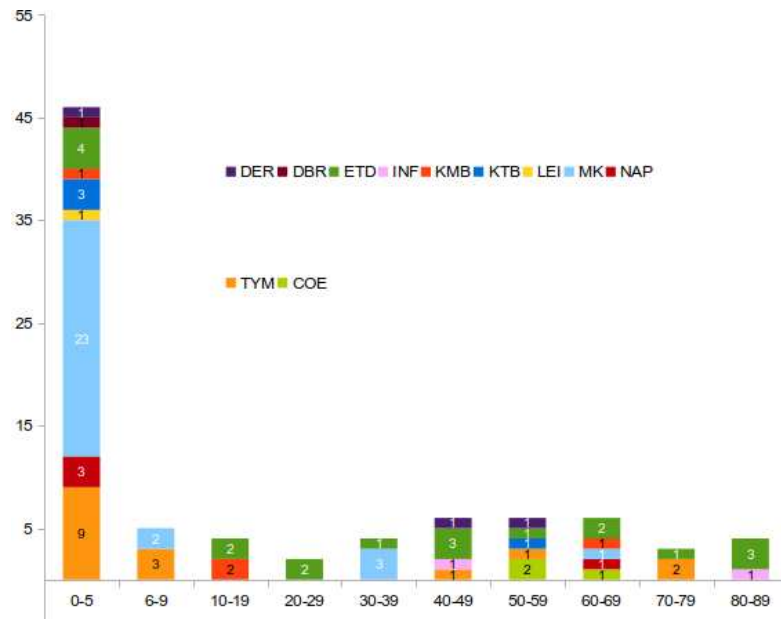


Figure 12. Serotype (n>2) distribution by age group.

* DER = *S. Derby*, COE = *S. Coeln*, DBR = *S. Dubrovnik*, ETD = *S. Enteritidis*, INF = *S. Infantis*, KMB = *S. Kambole*, KTB = *S. Kottbus*, LEI = *S. Leith*, MK = *S. Mikawasima*, NAP = *S. Napoli*, TYM = *S. Typhimurium*

During the peak in cases in early autumn (September and August), the largest number of cases were attributed to *S. Enteritidis*, whereas *S. Mikawasima* was the most dominant serotype in March, as seen in Figure 13, *S. Mikawasima* was also the only serotype isolated in February, with it being the cause of all 6 cases recorded during that month.

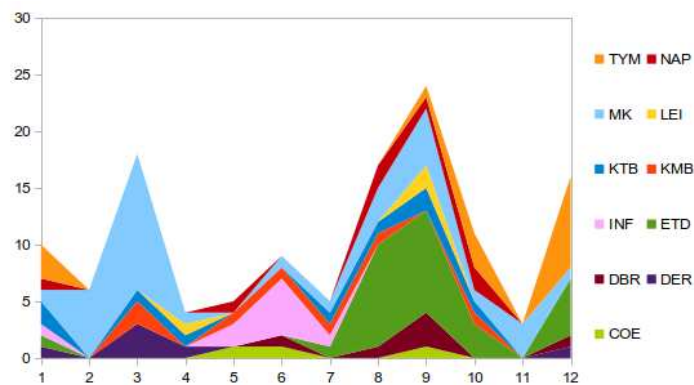


Figure 13. Isolates by serotype (n>2) by month.

*: DER = *S. Derby*, COE = *S. Coeln*, DBR = *S. Dubrovnik*, ETD = *S. Enteritidis*, INF = *S. Infantis*, KMB = *S. Kambole*, KTB = *S. Kottbus*, LEI = *S. Leith*, MK = *S. Mikawasima*, NAP = *S. Napoli*, TYM = *S. Typhimurium*

Serotype distribution varied somewhat by town, with more diversity in serotypes seen in larger towns with large amounts of tourism, such as Split. No compelling analysis of serotype distribution by town could be made due to low numbers in certain towns, such as Vrgorac, Brač, or Hvar. The largest proportion of one serotype with 2 or more cases found in one place was in Makarska with *S. Mikawasima* (N=4) comprising 30.769% of isolates with a known serotype (N=13) in that town; this is not statistically significant ($\chi^2=4.769$, $P=0.5737$).

When examining cases (N=135) where serotype, city, and month of isolation were all recorded, we found 10 instances where three or more isolates of one serotype were recorded in the same town, during the same month. These instances occurred in three towns: two in Makarska, 7 in Split, and one in Supetar. Four of these clusters, all occurring during the August-September peak in cases, involved *S. Enteritidis*. The remaining clusters involved other serotypes and most occurred during winter or spring. *S. Mikawasima* was involved in three clusters in Split with four cases in February, four in March, and three in September, as well as a cluster of three cases in Makarska, also in March. The one and only cluster of *S. Derby*, with three cases recorded in Split, also occurred in March. The cluster most suggestive of a possible local outbreak, was observed in December with 8 Split cases (61.54% of isolates in the city that month, 47.06% of all *S. Typhimurium* isolates in the sample) involving *S. Typhimurium*, the largest concentration of one serotype in one town during one month in the entire sample. When testing the number of *S. Typhimurium* cases reported in Split each month using a one-way chi-squared test there exists a statistically significant difference ($\chi^2=58$, $P<0.001$), which may be attributed to the cluster in December. All but one of the eight *S. Typhimurium* cases age involved children of 5 years of age or younger, which was the most common age seen in the other serotype clusters as well, apart from the March *S. Mikawasima* cluster in Makarska and in the one cluster in Supetar for which the ages were not recorded.

4.2. Antimicrobial resistance

Out of the total of 165 cases 161 included complete AMR-test-result data. 4 cases were missing these data. In total 70 isolates were found to be resistant to at least one antimicrobial; 8 of these isolates showed resistance to at least one agent from three different categories of antimicrobials, which is often used definition for multidrug resistance (MDR). 13 isolates out of the 165 were ESBL positive.

The antimicrobial against which the largest number (N=40) of isolates showed resistance was ampicillin (henceforth referred to as amoxicillin, as that is the more often used peroral formulation). Isolates resistant to ceftriaxone, chloramphenicol, were rarer, with only 5 isolates resistant to each (Table 2).

Table 2. Antimicrobial resistance results by tested antimicrobial (in cases with complete data (N=161) available).

Antibiotic	R	I	S
Amoxicillin	38		124
Amoxicillin-clavulanate	17		146
Ceftriaxone	5	3	156
Ceftazidime	16	1	147
Ciprofloxacin	27	1	136
Trimethoprim-Sulfamethoxazole	20		144
Chloramphenicol	5		158

* R = resistant, I = intermediate, S = susceptible.

In Figure 14 we see the proportion of resistant isolates for each antimicrobial in the study. The sex distribution of resistant isolates was entirely even (50% male, 50% female).

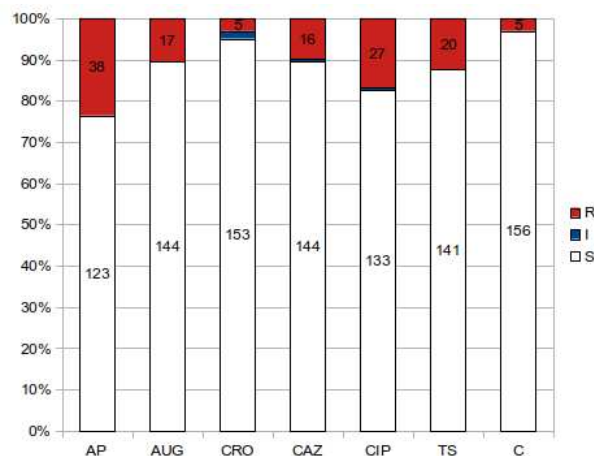


Figure 14. Total number (n) and percentage (%) of resistant isolates of *Salmonella* spp. for each antimicrobial in outpatients in Split-Dalmatia County (N=162).

47 isolates resistant to one or more antimicrobials had data available regarding age, with the vast majority (N=34) falling into the 5-10 age group, as seen in Figure 15.

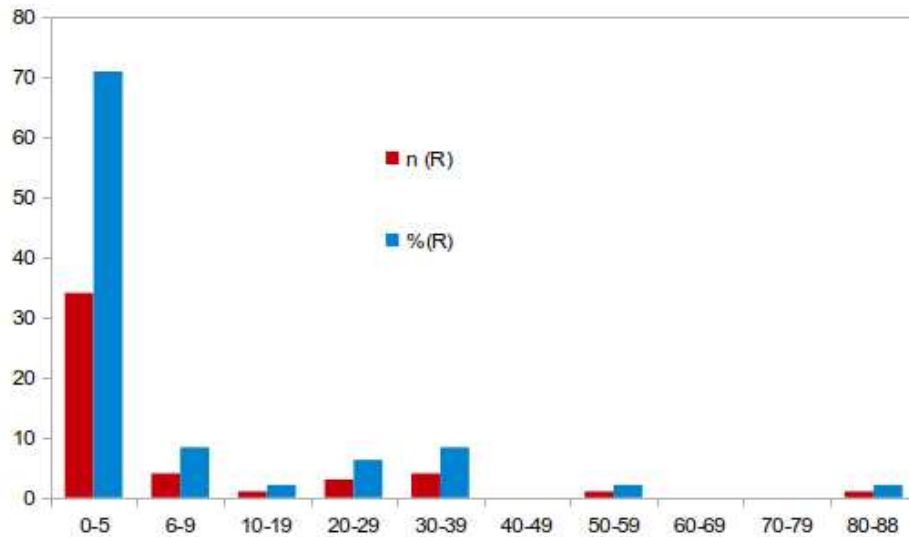


Figure 15. Number (n) and percentage (%) of resistant isolates in outpatients in Split-Dalmatia County (N=72) by age group.

Data on town was available for 64 of the resistant isolates. The largest number (N=46) of resistant isolates were recorded in Split, 11 of which were ESBL positive. The remaining two ESBL cases were recorded in Sinj, which also had the second highest number of resistant isolates with 6 (including ESBL positives) out of 16 isolates resistant to one or more antimicrobial drug. The largest number of resistant isolates was reported in Split. The number of resistant isolates for each town is shown in Figure 16. The highest rate of antimicrobial resistance, when accounting for town population, however, was recorded in Jelsa, followed by Makarska and Split. No cases exhibiting resistance to tested antimicrobials were recorded in Brač (incl. Supetar), Omiš, or Vrgorac.

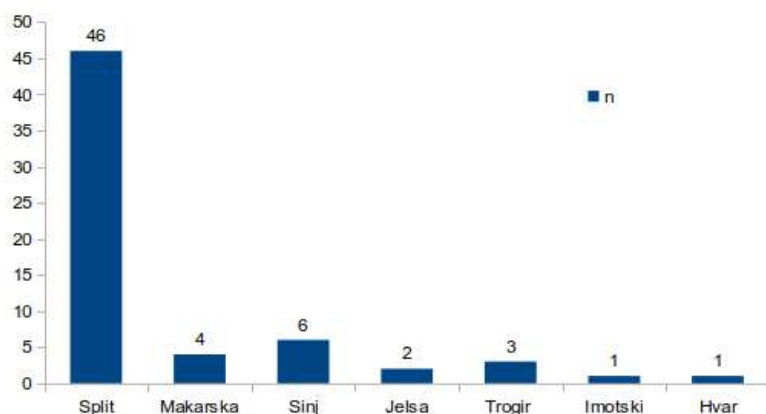


Figure 16. Number (n) of isolates with resistance to one or more of the tested antimicrobials.

Month of isolation was recorded for 66 of the isolates with resistance against at least one antimicrobial and the distribution of these over twelve months is shown on Figure 17.



Figure 17. Temporal distribution of isolates with resistance to one or more tested standard antimicrobials in the sample.

Serotyping data were available for 63 out of the 70 resistant isolates the majority of which were, as is the case for all isolates regardless of resistance status, *S. Mikawasima* (N=29) with 82.857% of isolates of this variant being resistant to one or more antimicrobials, and as seen in Figure 20., this variant makes up the majority of isolates resistant to penicillins (amoxicillin, amoxicillin-clavulanate) and cephalosporins (ceftriaxone, ceftazidime). *S. Enteritidis* isolates were mostly fully susceptible (N=23) to all tested antimicrobials, with four isolates being resistant against ciprofloxacin alone and two against both ciprofloxacin and trimethoprim-sulfamethoxazole. *S. Typhimurium* isolates had AMR data available in 16 out of 17 cases, and 13 out of the 16 (81.25%) were resistant to one or more antimicrobials. Notably, the cluster of cases of *S. Typhimurium* seen in Split in December 2022 showed, near identical AMR profiles: all but one December 2022 *S. Typhimurium* isolates from Split (N=12) were resistant to ciprofloxacin (and fully susceptible to other tested antibiotics), with the one ciprofloxacin sensitive isolate being, unlike the rest, amoxicillin-resistant. Also worth mentioning that all nine *S. Infantis* isolates were resistant to at least one antimicrobial agent, most (N=7) or 77.778% were resistant against ciprofloxacin, or trimethoprim-sulfamethoxazole (N=5; 55.56%), with one isolate resistant to both and another being resistant against amoxicillin in addition to ciprofloxacin.

Of the eight isolates resistant to multiple (three or more) classes of antimicrobial, 3 of which were also marked as ESBL positive. All multidrug-resistant isolates, for which serotype was recorded (N=4), were serotype *S. Mikawasima*. All 8 multidrug-resistant isolates were also resistant to amoxicillin. The least resistance among the multi-resistant isolates was against ciprofloxacin, with only one such isolate of unknown serotype, resistant against amoxicillin

and chloramphenicol in addition to ciprofloxacin. Ceftriaxone resistance was similarly rare, with one isolate exhibiting resistance against ceftriaxone in addition to ceftazidime and chloramphenicol. Most frequent resistance combination (N=4) was combined resistance to amoxicillin, trimethoprim-sulfamethoxazole, and chloramphenicol. The town was recorded for all except for one of the multi-resistant isolates with all except one case being recorded in Split.

Every one of the 13 ESBL isolates in the study population was serotyped as *S. Mikawasima*. All ESBL positive cases occurred in the “0-5”-age-group. The vast majority (N=11; 84.61%) were recorded in Split, with the remaining two cases being from Sinj. The difference in sex distribution among ESBL-positive cases was not statistically significant ($\chi^2=0.692$, $P=0.405$). A large proportion (76.92%) of cases involving ESBL isolates (N=10), were recorded during the first four months of the year with a distinct peak occurring during February and March; all except one case between January and April occurred in Split. In addition to the seven commonly tested antimicrobials, three ESBL isolates were tested for other agents such as meropenem, imipenem, gentamycin, and amikacin. One isolate from a Split infant was found to be resistant to gentamycin and amikacin, in addition to the commonly tested penicillins (amoxicillin-clavulanate) and ceftriaxone. No carbapenem resistance was detected in the two isolates tested for imipenem and meropenem.

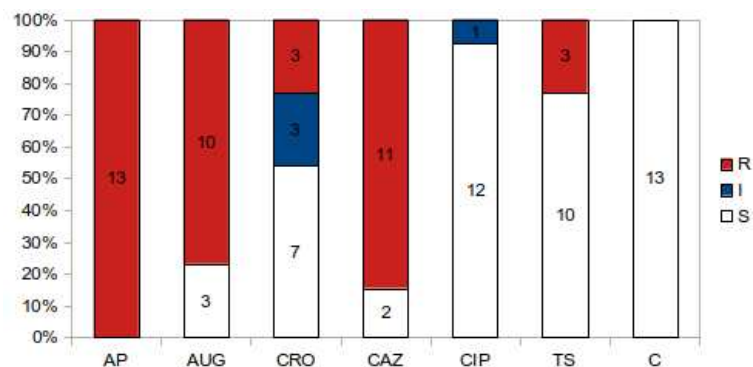


Figure 18. Number and proportion of ESBL (*S. Mikawasima*) isolates from outpatients in SCD in 2022 by resistance to different antimicrobial agents.

*S = susceptible, R = resistant, ATU = Area of Technical Uncertainty

AP = ampicillin, CRO = ceftriaxone, CAZ = ceftazidime, AUG = amoxicillin-clavulanate, C=chloramphenicol, TS = trimethoprim-sulfamethoxazole, CIP = ciprofloxacin.

5. DISCUSSION

We found that the total number of non-typhoidal *Salmonella* infections in outpatients in Split-Dalmatia County in 2022 was 165, most of which occurred in children 5 years old and younger. As expected, most of the cases were reported in the County capital Split, but more surprisingly, the highest number of cases in proportion to town population occurred in Jelsa, a very small town. The most frequently identified serotype was *S. Mikawasima*, as opposed to the nationally and EU-wide most common *S. Enteritidis*. This mirrors the findings of Carev et al. (84). Interestingly, according to our data, *S. Mikawasima* also harbored antimicrobial resistance more often than other serotypes, and was the only serotype showing ESBL expression.

Among outpatient isolates with AMR data available in SCD in 2022, we found that, prevalence of resistance against amoxicillin, amoxicillin-clavulanate, and ceftazidime, were higher in the SDC in comparison with the national prevalence levels as regarding reported by ISKRA and the Croatian Academy of the Sciences for that year (126). However, the ranges of local results for the national data from 2022 were wide and all SCD data fall within these, meaning no true difference may exist. No SDC-specific results were provided in the report, but a retrospective study about *Salmonella* spp. in the SDC by Carev et al. (84) found resistance levels not significantly (“N-1”-Chi-squared, $P > 0.001$ for all) dissimilar to either the ISKRA report or our results (126). The minor differences between the findings of Carev et al. (84) and our paper may be explained by the fact that their data included also hospitalized patients, isolates from whom may have differing antimicrobial resistance profiles.

Between our results and those of Carev et al. (84) there was no significant difference regarding ESBL-producer prevalence. Much like all ESBL-positive isolates in our study, every ESBL-positive isolate found between 2020-2022 by Carev et al. (84) was of serotype *S. Mikawasima*. Some of these isolates may be related to the strain(s) responsible for the hospital outbreak of ESBL-expressing *Salmonella Mikawasima* in the neonatology ward of the University Hospital of Split (UHS) during the COVID-19-pandemic, described by Novak et al. in a recent paper (84).

Between national and European comparison data, as well as County-specific findings from recent papers by Carev et al. and Novak et al., both in 2024, it appears that the high prevalence of *S. Mikawasima* is a fairly unique characteristic of the SDC, possibly suggesting the presence of a common source in the area (83,84). Further research aimed towards identifying such a source may be warranted, especially as Carev et al. suggest, based on comparison with 2005-2007 data, that the emergence of this variant is fairly recent (84).

Besides the prevalence and high levels of antimicrobial resistance among *S. Mikawasima*, we also found that in the outpatient population of the SCD, the variant appears, at least in the 2022 data, to have a few other interesting characteristics: firstly, the variant was more common than other serotypes among 0-5-year-olds. Secondly, an unanticipated (based on available data on usual epidemiologic data from the EU and from the SCD between 2020-2022) peak in salmonellosis cases occurred in March 2022 in Split, comprising nearly entirely of *S. Mikawasima*, possibly indicating a common source.

Another attention-worthy finding in our study was the high prevalence (in proportion to town population) of salmonellosis cases in outpatients in the small town of Jelsa, as well as in Makarska, although there were relatively few cases in numbers making conclusions hard to draw from this data. We may have also identified a possible small-scale outbreak of *S. Typhimurium* in Split in December 2022, as suggested also by the similar antimicrobial resistance patterns of this variant seen in that town during that month.

It is important to note that due to the study and sample type, as well as the fact that complete data on all parameters was not available for all isolates, there is a comparatively high risk of bias. *Salmonella* infection surveillance in outpatients in SCD may underestimate the true number of cases due to the nonspecific and mild nature of the disease, leading to patients not informing their doctors about their illness. The possibility of misdiagnosis and subsequent lack of reporting/microbiological investigation on the part of physicians who were sought out by salmonellosis patients is also present. Unfortunately, physicians reporting the cases may not also have followed the proper course of action in reporting the illness, even with the correct diagnosis made. Poor sample quality/handling, laboratory error and missing or incompletely/incoherently reported data are also possibilities. Our paper also focused on outpatients, so some more severe cases, possibly with more concerning AMR profiles as some evidence suggests, were perhaps not accounted for (61). The availability and quality of comparative data currently available, on salmonellosis rates, serotype distribution, and several patient parameters on the Croatian and European level was not ideal and presents some problems for determining the significance of our findings.

Considering the above more rigorous, larger scale research focused on a wider timeframe may be warranted to fully quantify and qualify the special characteristics of human pathogenic *Salmonella* spp. in SCD outpatients.

6. CONCLUSIONS

We conclude that among outpatients in the SCD in 2022, *Salmonella* spp. exhibited epidemiological and antimicrobial resistance patterns typical of the species in Europe. Some unusual characteristics include the high prevalence of *S. Mikawasima*, especially among young children, and during unusual times of the year, as well as the high level of antimicrobial resistance and ESBL expression within this variant.

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8. SUMMARY

Objectives: Our goal was to describe the epidemiological characteristics and antimicrobial resistance patterns of non-typhoidal *Salmonella* spp. in the outpatient population of Split-Dalmatia County in 2022.

Materials and methods: We analyzed data from an anonymized computerized database including patient data on age, sex, town of residence, *Salmonella* serotype, and antimicrobial resistance.

Results: 165 outpatients with non-typhoidal *Salmonella* infections were recorded in Split-Dalmatia County in 2022. The most common age group affected was children aged 0 to 5 years. The largest number of cases was reported in Split, and the incidence was highest in Jelsa. The most common serotype among outpatients in Split-Dalmatia County in 2022 was *S. Mikawasima*. The most common antimicrobial resistance was against amoxicillin (24,85% of isolates), and the least resistance was found against ceftriaxone (3%), and chloramphenicol (3%). There were 13 EBSL-positive isolates, all *S. Mikawasima*.

Conclusion: In comparison with European and national epidemiologic data the high prevalence of *S. Mikawasima* in Split-Dalmatia County is a notable finding. Recent research focusing on *Salmonella* spp. in the county has had similar findings, which may imply that the abundance of this serotype is due to some local source. Also, of interest may be the findings that, the serotype *S. Mikawasima* in particular is common in young children under 6 years old, and that a relatively large proportion of *S. Mikawasima* cases (37%) in the county in 2022 were EBSL-positive.

9. CROATIAN SUMMARY

Naslov: Epidemiološke i mikrobiološke značajke *Salmonella* spp. infekcije u ambulantnih bolesnika u Splitsko-dalmatinskoj županiji 2022. godine: presječna studija

Ciljevi: Naš je cilj bio opisati epidemiološke značajke i obrasce antimikrobne rezistencije netifusne *Salmonella* spp. u ambulantnih bolesnika u Splitsko-Dalmatinske županije 2022.

Materijali i metode: Analizirali smo podatke iz anonimizirane računalne baze podataka uključujući podatke o dobi, spolu, mjestu stanovanja bolesnika, serotipu i antimikrobnoj otpornosti salmonela.

Rezultati: Tijekom 2022. godine u Splitsko-dalmatinskoj županiji (SDŽ) zabilježeno je 165 ambulantnih pacijenata s netifusnim infekcijama salmonelom. Najčešće oboljela dobna skupina bila su djeca od 0 do 5 godina. Najveći broj oboljelih zabilježen je u Splitu, a incidencija je bila najveća u Jelsi. Najčešći serotip među ambulantnim pacijentima u Splitsko-dalmatinskoj županiji u 2022. je bio *S. Mikawasima*. Najviše izolata *Salmonella* spp. je bilo rezistentno na amoksicilin (24,85% izolata), a najmanja na ceftriakson (3%), i na kloramfenikol (3%). Bilo je 13 EBSL-pozitivnih izolata, svi serotipa *S. Mikawasima*.

Zaključci: U usporedbi s Europskim i nacionalnim epidemiološkim podacima, visoka prevalencija *S. Mikawasime* u SDŽ-u značajan je nalaz. Nedavna istraživanja usmjerena na *Salmonella* spp. u županiji su imala slične nalaze, što može značiti da je prevladavanje ovog serotipa posljedica prisutnosti u nekom lokalnom izvoru. Također bi mogli biti zanimljivi nalazi da je serotip *S. Mikawasima* čest u djece mlađe od 6 godina i da je relativno velik udio slučajeva *S. Mikawasime* u Županiji 2022. godine bio EBSL pozitivan.