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# Human bile microbiota: A retrospective study focusing on age and gender

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ABSTRACT

*Aims:* The emerging biliary colonization of microorganisms in patients with biliary diseases may be devastating. Recent evidence suggests that age and gender may influence changes in the microbial composition of gut microbiota. To study the relationship between these parameters on bile microbiota, we retrospectively reviewed positive bile cultures following an endoscopic retrograde cholangiopancreatography (ERCP) in a QA-certified academic surgical unit of a single institution.

*Methods:* 449 positive bile cultures from 172 Italian patients with diseases of the biliopancreatic system hospitalized from 2006 through 2017 were investigated for aerobic, anaerobic, and fungal organisms. The patients were stratified into four age intervals (22–66, 67–74, 75–81, and 82–93 years) and followed up for five years.

*Results:* Gram-positive bacteria (GPB) was negatively associated with age only in multivariate analysis ($R_{partial} = -0.114$, $p = 0.017$), with younger patients prone to harbor GPB and older patients likely to have Gram-negative bacteria (GNB). There was a definite link with the male gender using both univariate and multivariate analysis ($p < 0.001$). *Enterococcus* spp. was the most common strain identified in patients with GPB except for patients aged 67–74 years for male (95.2%) and female (80.9%) patients. *Escherichia coli* and *Klebsiella* spp. were most frequent than others in every group analyzed. Analogous results were found for bacteria Non-fermenting Gram-negative bacilli (NFGNB), such as *Pseudomonas* spp. and *Stenotrophomonas* spp. apart of the 2nd quartile.

*Conclusions:* Our study strengthens the bond of age and gender with bile microbiota composition and suggests that further investigations may be required in targeting the aging microbiome. Other studies should also focus on Mediterranean epidemiological characteristics and antibiotic resistance surveillance system strategies



*Abbreviations:* CBD, common bile duct; ED, early death; EMR, electronic medical records; ERCP, endoscopic retrograde cholangiopancreatography; ESCMID, European Society of Clinical Microbiology and Infectious Diseases; EUCAST, European Committee on Antimicrobial Susceptibility Testing; GNB, gram-negative bacteria; GNBNI, gram-negative bacilli not identified; GPB, gram-positive bacteria; MDR, multi-drug resistance; NFGNB, non-fermenting gram-negative bacilli; PCR, polymerase chain reaction; QA, quality assurance; SD, standard deviation; STROBE, Strengthening the Reporting of Observational Studies in Epidemiology.

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[1] Both authors contributed equally to the manuscript. N. Serra is a mathematician and performed the statistical analysis and interpretation of data. Dr. P. Di Carlo is a member of the European Society of Clinical Microbiology and Infectios Diseases (ESCMID).

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## Introduction

The human gut microbiota composition can be altered by diet, age, antibiotics, and disease. Both mucosal barrier depletion and bacterial diversity loss are primary abnormalities in some forms of inflammatory bowel disease [1]. Recent evidence suggests that age and gender may influence changes in the microbial composition of gut microbiota [2–4]. The increased aging of the population may indicate that the elderly are in good health. Several studies have evidenced an increased rate of acute admissions for this age group in the last two decades [5–8]. Obstruction of the biliary system by malignancies, anastomotic stenosis after liver transplantation, chronic pancreatitis, or gallstones are some of the most common causes for elective or emergency endoscopic retrograde cholangiopancreatography (ERCP) in the elderly [9–12]. Biliary tract infection is a common cause of bacteremia and is associated with high morbidity and mortality. It occurs mainly in patients with comorbidities [10,13].

Bile was considered a sterile fluid, but recent reports indicate that a microbial ecosystem does exist in patients with and without hepatobiliary disorders [8,10,13–17]. The biliary system is in continuous contact with the gut microbiota, and microbial products have recently been proposed as potential triggers for biliary diseases [18,19]. The influences of gender and aging on the gut microbiota composition and function have been described, but no comprehensive data are present for the bile [20,21]. Recently, correlations between gut microbiota and aging were investigated in several studies showing that senescence can affect gut bacterial species' composition and metabolic functions [22–24].

Further, this alteration can be associated with numerous gut-related diseases in the host [21,25]. These studies may be the premise to study microbiologic colonization in perinatology, interpret epigenetic data in inflammatory bowel disease, and design new drugs in the nearest future [2–4,26,27]. In line with these studies, we carried out a cross-sectional survey of human bile samples to assess microbiota typing.

## Materials and methods

This study is a retrospective investigation of patients with positive bile cultures identified from the database using institutional electronic medical records (EMRs). All procedures performed in our study involving human participants were carried out by the institutional and national research committee's ethical standards. Informed consent was signed by all patients included in this study. Anonymity was guaranteed for all patients. No economic incentives were offered or provided for participation in this study. The analysis was performed following the ethical considerations of the Helsinki Declaration. This study was approved by the local University Ethics Committee (UIN3220). All participants granted consent to publish our data in anonymized form.

This study was conducted among hospitalized adult patients using the International Classification of Diseases (ICD-10). The hospital setting is the Policlinic University Hospital of Palermo, Italy. Data were collected from January 2006 to January 2017. The biliary and pancreatic system diseases were extracted from medical charts and double-checked by two team specialists. The study population consisted of Italian patients with a positive culture of bile samples collected during ERCP from patients harboring hepatobiliary disease at an external quality assurance (QA)-certified General Surgery and Emergency Academic Unit. Inclusion criteria: ERCP performance with at least one positive bile sample for fungal o bacteria and/or cyto- and/or histopathological examination. In the period of patients' recruitment, the number of endoscopic procedures carried out was 250/year on average.

The patients who underwent ERCP were subjects during the first surgery. Patients were subjects readmitted at our unit and outpatients emergency individuals from other hospitals of the Sicilian region (50% of endoscopic procedures per year). As previously reported, the commonly observed surgical procedures included acute cholecystitis, post-operative complications of biliary surgery, obstruction (cholelithiasis, choledocholithiasis, primary sclerosing cholangitis), and malignancies of the pancreas and the biliary tract [2–4]. Our active surveillance cultures, including bile culture, have been carried out in the Surgical Emergency Unit, especially at a higher risk of life-threatening complications such as intra-abdominal infection [28].

Moreover, as a surveillance bile culture, bile specimens were collected at either preoperative biliary drainage procedure or surgery, especially in malignant tumors [2–4].

Medical comorbidities such as diabetes and cardiovascular disease mirrored those reported in the general population according to age and sex during the study period [4].

The bile collection was obtained by introducing an ERCP standard 5 Fr catheter (Olympus Medical Systems Co. Tokyo, Japan) deeply into the common bile duct (CBD) before the contrast medium injection. In sepsis due to plastic stent obstruction, the biliary sample was obtained immediately after the endoscopic stent removal.

Four hundred forty-nine bile samples showed microorganismal isolates identified according to international guidelines and microbiological standards [29]. The 449 positive bile samples were obtained from 172 inpatients (93 males and 79 females, 22–93 years-old, $71 \pm 13.9$ years, mean $\pm$ standard deviation). Concerning the number of samples for patients, multiple samples were considered for patients at admission. In particular, the mean of the number of samples collected was equal to 2.6. In different results by two collected samples, additional samples were collected until the more expressed isolate was fully identified. In this way, we reduced the possible biases both in the identification of isolates and statistical analysis.

To evaluate the role of age and gender, we defined our subgroups considering age (Group A, Group B, Group C, and Group D) and gender (Group F (females) and Group M (males), as shown in Table 1. Notably, we considered a cut-off to define age groups' quartile values: 66, 74, and 81 years. In this way, we divided the age range into four equal parts identifying the following intervals: 22–66, 67–74, 75–81, 82–93 years.

The EMRs, endoscopic imaging, and histopathological data were abstracted to standardized forms. The following data were extracted: age, gender, date of procedure, ERCP results, histopathology, predisposing factors, clinical, endoscopic, and radiological features. All procedures were performed under strict quality control parameters and optimized computational analytics [7]. Bacterial identification and antimicrobial susceptibility testing were carried out using either the Phoenix Automated Microbiology System (Becton Dickinson Diagnostic Systems, Sparks, United States) or the Vitek-2 System (Bio-Mérieux, Marcy l'Etoile, France). According to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints, the antimicrobial sensitivity test of the strains was determined as previously reported [30]. *Candida* spp. were also identified by conventional morphological and biochemical methods, as previously reported [31]. We categorized the patients into three groups: ED (early death or death within six months), non-ED-1 (alive into the field of 6–12 months), and non-ED-2 (alive for more than 12 months).

**Table 1**
Distribution of study participants according to age and gender.

| Group | Mean Age | Age range | Mean Age at recruiting | Gender (M%/F%) | Samples | Patients |
|---|---|---|---|---|---|---|
| All Groups | 71.0 ± 13.9 | 22–93 | 64.3 ± 14.7 | 54.1 / 45.9 | 449 | 172 |
| *Age subgroups* | | | | | | |
| Group A | 54.3 ± 11.4 | 22–66 | 48.4 ± 11.6 | 53.1 / 46.9 | 113 | 49 |
| Group B | 70.5 ± 2.4 | 67–74 | 64.2 ± 3.1 | 52.6 / 47.4 | 110 | 38 |
| Group C | 77.9 ± 2.1 | 75–81 | 71.6 ± 2.1 | 56.8 / 43.2 | 118 | 44 |
| Group D | 86.0 ± 3.0 | 82–93 | 79.3 ± 3.8 | 53.7 / 46.3 | 108 | 41 |
| *Gender subgroups* | | | | | | |
| Group M | 71.7 ± 13.1 | 24–92 | 65.5 ± 12.9 | 54.1% | 262 | 93 |
| Group F | 70.2 ± 14.7 | 22–93 | 63.9 ± 14.9 | 45.9% | 187 | 79 |

**Table 2**
Univariate and multivariate linear correlation analysis between Gram typing with age and gender.

| *Parameters* | Univariate analysis | Multivariate analysis |
|---|---|---|
| *Correlations* | *R (p-value)* | $R_{partial}$ *(p-value)* |
| Gram (+/−)/Age | −0.058 (0.224) | $R_{partial}$ = −0.116; p-value = 0.015* |
| Gram (+/−)/Gender | 0.469 (<0.0001)* | $R_{partial}$ = 0.478; p-value < 0.001* |

* = significant test; R = Pearson's linear correlation coefficient; *R_partial*= the partial correlation coefficient is the coefficient of correlation of the variable with the dependent variable, adjusted for the effect of the other variables in the mode.

## Statistical analysis

We used MATLAB software version 2008 (MathWorks, Natick, MA, USA) for statistical analysis. Data are presented as numbers and percentages for categorical variables. Continuous data are expressed as the mean ± standard deviation (SD) or median with the Interquartile Range (IQR). We performed the chi-square test and Yates's continuity correction or Fisher's exact test to compare the differences between two percentages or proportions for independent data. A multiple comparison chi-square test was used to define significant differences among percentages. In this case, if the chi-square test was significant ($p < 0.05$), a post hoc Z-test was performed to identify to locate the highest or lowest significance of the bacterial presence. Finally, univariate and multivariate linear correlation analyses were also performed. In this case, the test on Pearson's linear correlation coefficient R was performed with Student's t-test, under the null hypothesis of Pearson's linear correlation coefficient of R = 0. Accordingly, we considered the dependent variable as Gram typing and the independent variables as Age and Gender. In particular, concerning Gram typing and gender, we defined the probability distributions, assigning the value of 1 in the presence of Gram-positive bacteria (GPB) and the amount of 0 in the presence of Gram-negative bacteria (GNB) as well as 1 in the presence of male and 0 in the presence of female subjects. All tests with p-value (p) < 0.05 were considered significant.

## Results

We analyzed possible correlations between the Gram typing with age and gender (Table 2). The significant predictor of GNB was the age in multivariate analysis, while the significant predictor of GPB was the male gender performing well in both univariate and multivariate analyses. We found that young patients had more probability to harbor GPB, while old patients had more chance to have GNB (Rpartial = −0.116, p = 0.015 in multivariate analysis). Moreover, GPB was most often seen in males, while GNB was mainly found in females (R = 0.469 p < 0.001 in univariate analysis, and Rpartial = 0.478, p < 0.001 in multivariate analysis).

Fig. 1 shows the total bile sample isolated divided by single isolates and groups. The isolates have been identified by Gram staining technical and biochemical analysis (FGNB and NFGNB). We subdivide in Tables 3 and 4 the FGNB into two groups, including the 'Enterobacteriaceae family' and the 'no Enterobacteriaceae' family.

**Table 3**
Statistical analysis of the 449 bile isolates divided by Gram stain and gender.

| Isolates | All Patients (%) 22–93 years | Males (%) 24–92 years | Females (%) 22–93 years |
|---|---|---|---|
| Gram (+) | 9.3 = 42/449 | 8.0 = 21/262 | 11.2 = 21/187 |
| Gram (−) | 89.3 = 401/449 | 90.8 = 238/262 | 87.2 = 163/187 |
| Gram (+) | | | |
| *Enterococcus* spp. | 88.1 = 37/42 * | 95.2 = 20/21 * | 80.9 = 17/21) * |
| *Streptococcus* spp. | 9.5 = 4/42 ** | 4.8 = 1/21 ** | 14.3 = 3/21 |
| *Staphylococcus* spp. | 2.4 = 1/42 ** | 0.0 = 0/21 ** | 4.8 = 1/21 ** |
| Gram (−) | | | |
| NFGNB | 65.1 = 261/401 | 64.3 = 153/238 | 66.3 = 108/163 |
| FB | 34.9 = 140/401 | 35.7 = 85/238 | 33.7 = 55/163 |
| FB | | | |
| *Enterobacteriaceae* | 99.3 = 139/140 | 98.8 = 84/85 | 100 = 55/55 |
| No *Enterobacteriaceae* | 0.7 = 1/140 | 1.2 = 1/85 | 0.0 = 0/55 |
| *Enterobacteriaceae* | | | |
| *E. coli* | 41.7 = 58/139 * | 36.9 = 31/84 * | 49.1 = 27/55 * |
| *Klebsiella* spp. | 31.6 = 44/139 * | 33.3 = 28/84 * | 29.1 = 16/55 * |
| *Citrobacter* spp. | 10.1 = 14/139 | 10.7 = 9/84 | 9.1 = 5/55 |
| *Enterobacter* spp. | 10.8 = 15/139 | 11.9 = 10/84 | 9.1 = 5/55 |
| *Proteus* spp. | 2.2 = 3/139 ** | 2.4 = 2/84 ** | 1.8 = 1/55 ** |
| *Morganella* spp. | 0.7 = 1/139 ** | 0.0 = 0/84 ** | 1.8 = 1/55 ** |
| *Serratia* spp. | 2.2 = 3/139 ** | 3.6 = 3/84 ** | 0.0 = 0/55 ** |
| *Pantoea* spp. | 0.7 = 1/139 ** | 1.2 = 1/84 ** | 0.0 = 0/55 ** |
| No *Enterobacteriaceae* | | | |
| *Aeromonas* spp. | 100 = 1/1 | 100 = 1/1 | 0.0 = 0/1 |
| NFGNB | | | |
| *Pseudomonas* spp. | 47.5 = 124/261 * | 45.7 = 70/153 * | 50.0 = 54/108 * |
| *Stenotrophomonas* spp. | 19.5 = 51/261 * | 20.3 = 31/153 * | 18.5 = 20/108 * |
| *Alcaligenes* spp. | 7.3 = 19/261 | 7.8 = 12/153 | 6.5 = 7/108 |
| *Acinetobacter* spp. | 6.9 = 18/261 | 7.8 = 12/153 | 5.6 = 6/108 |
| *Achromobacter* spp. | 5.4 = 14/261 ** | 5.2 = 8/153 | 5.6 = 6/108 |
| *Sphingobacterium* spp. | 0.4 = 1/261 ** | 0.0 = 0/153 ** | 0.9 = 1/108 ** |
| *Brevundimonas* spp. | 1.9 = 5/261 ** | 2.0 = 3/153 ** | 1.8 = 2/108 ** |
| *Delftia* spp. | 1.1 = 3/261 ** | 0.6 = 1/153 ** | 1.8 = 2/108 ** |
| *Elizabethkingia* spp. | 1.1 = 3/261 ** | 1.3 = 2/153 ** | 0.9 = 1/108 ** |
| *Sphingomonas* spp. | 1.1 = 3/261 ** | 0.6 = 1/153 ** | 1.8 = 2/108 ** |
| GNBNI | 7.7 = 20/261 | 8.50 = 13/153 | 6.5 = 7/108 |
| Fungus | | | |
| Candida | 1.3 = 6/449 | 1.1 = 3/262 | 1.6 = 3/187 |

F = female; M = male, FB = fermenting bacteria; NFGNB = Non-fermenting Gram-negative bacilli, GNBNI = Gram-negative bacilli not identified, * = microbial species with a significant high rate of occurrence, ** = microbial species with a significant low rate of occurrence.

Table 3 shows a significant difference among all microbiological species (p < 0.0001 by multivariate analysis) considering all 449 samples. In particular, the most frequent bacteria identified by post hoc Z-test were *Pseudomonas* spp. (27.6%, p < 0.001), *E. coli* (12.9%, p < 0.001), *Stenotrophomonas* spp. (11.4%, p < 0.001), *Klebsiella* spp. (9.8%, p < 0.001), *and Enterococcus* spp. (8.2%, p < 0.001). On the other hand, the lowest isolates were *Brevundimonas* spp. (1.1%, p = 0.041), *Streptococcus* spp. (0.9%, p < 0.001), *Proteus* spp. (0.7%, p < 0.001), *Serratia* spp. (0.7%, p < 0.001), *Sphingomonas* spp. (0.7%, p < 0.001), *Delftia* spp. (0.7%, p < 0.001), *Elizabethkingia* spp. (0.7%,

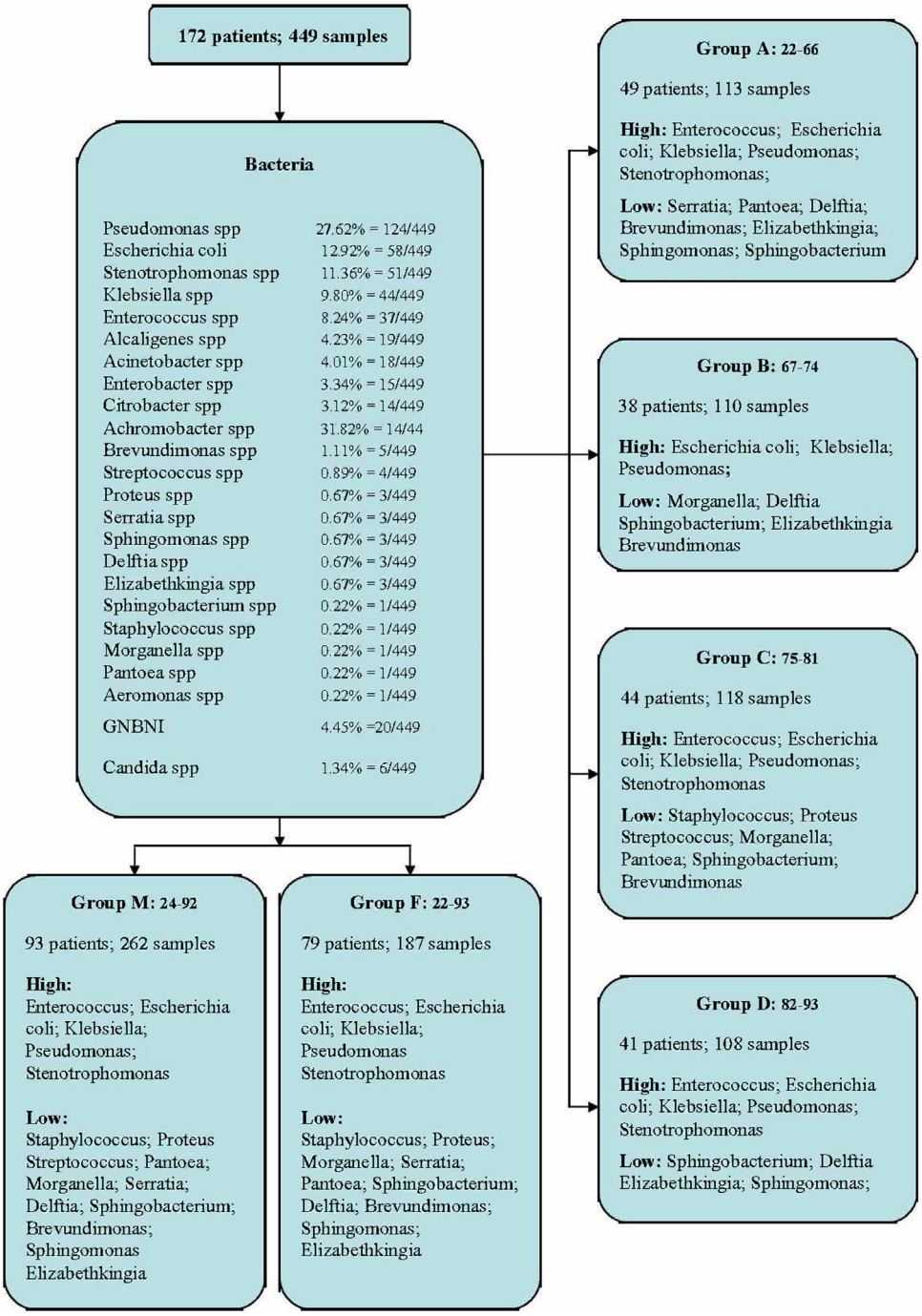


**Fig. 1.** Block diagram of the study.

p < 0.001), *Sphingobacterium* spp. (0.2%, p < 0.001), *Staphylococcus* spp. (0.2%, p < 0.001), *Morganella* spp. (0.2%, p < 0.001), *Pantoea* spp. (0.2%, p < 0.001), *Aeromonas* spp. (0.2%, p < 0.001), and the fungal strain represented by *Candida* spp. (1.3%, p = 0.003).

Also, we considered bacteria classes such as Gram+, Enterobacteriaceae, and no Enterobacteriaceae and observed (column 2) that the most frequent identified bacteria were *Pseudomonas* spp. (47.5% = 124/449, p < 0.001) and *Stenotrophomonas* spp. (19.5%, p < 0.001) among NFGNB bacteria; *E. coli* (41.7%, p < 0.001) and, *Klebsiella* spp. (31.6%, p < 0.001), among *Enterobacteriaceae*; and *Enterococcus* spp. (88.1%, p < 0.001) among GPB. On the other hand, the lowest isolates were *Achromobacter* spp. (5.4%, p = 0.031), *Brevundimonas* spp. (1.9%, p < 0.001), *Delftia* spp. (1.1%, p < 0.001), *Sphingomonas* spp. (1.1%, p < 0.001), *Elizabethkingia* spp. (1.1%, p < 0.001), and *Sphingobacterium* spp. (0.4%, p < 0.001) among NFGNB bacteria. *Proteus* spp. (2.2%, p < 0.001), *Serratia* spp. (2.2%, p < 0.001), *Morganella* spp. (0.7%, p < 0.001), and *Pantoea* spp. (0.7%, p < 0.001) among *Enterobacteriaceae*. Finally, *Streptococcus* spp. (9.5%, p = 0.005), and *Staphylococcus* spp. (2.4%, p < 0.001) among GPB.

The analysis of 449 bile samples divided by gender are shown in Table 3. The analysis into groups showed that among the GPB, the most common strain was *Enterococcus* spp. (Group M: 95.2%, p < 0.001; Group F: 80.9%, p < 0.001) while *Staphylococcus* spp. was the less represented strain (Group M: 0.0%, p = 0.005; Group F: 4.8%, p = 0.014). Conversely, among *Enterobacteriaceae* (GNB), the most frequently identified strains were *E. coli* (Group M: 36.9%, p < 0.001;

**Table 4**
Statistical analysis of the 449 bile isolates by age groups with all microorganisms represented as genera (spp.) apart from *E. coli*.

| Isolates | Group A (%) 22–66 years | Group B (%) 67–74 years | Group C (%) 75–81 years | Group D (%) 82–93 years |
|---|---|---|---|---|
| Gram(+) | 8.0 = 9/113 | 6.4 = 7/110 | 14.4 = 17/118 | 8.3 = 9/108 |
| Gram(–) | 92.0 = 104/113 | 91.8 = 101/110 | 84.7 = 100/118 | 88.9 = 96/108 |
| Gram(+) | | | | |
| *Enterococcus* spp | 88.9 = 8/9 * | 71.4 = 5/7 | 88.2 = 15/17 * | 100 = 9/9 * |
| *Streptococcus* spp | 11.1 = 1/9 | 28.6 = 2/7 | 5.9 = 1/17 ** | 0.0 = 0/9 |
| *Staphylococcus* spp | 0.0 = 0/9 | 0.0 = 0/7 | 5.9 = 1/17 ** | 0.0 = 0/9 |
| Gram(-) | | | | |
| NFGNB | 67.3 = 70/104 | 63.4 = 64/101 | 61 = 61/100 | 68.7 = 66/96 |
| FB | 32.7 = 34/104 | 36.6 = 37/101 | 39 = 39/100 | 31.3 = 30/96 |
| FB | | | | |
| *Enterobacteriaceae* | 100 = 34/34 | 97.3 = 36/37 | 100 = 39/39 | 100 = 30/30 |
| No *Enterobacteriaceae* | 0.0 = 0/34 | 2.7 = 1/37 | 0.0 = 0/39 | 0 = 0/30 |
| *Enterobacteriaceae* | | | | |
| *E. coli* | 47.1 = 16/34 * | 38.9 = 14/36 * | 41.0 = 16/39 * | 40 = 12/30 * |
| *Klebsiella* spp. | 32.3 = 11/34 * | 25.0 = 9/36 | 35.9 = 14/39 * | 33.3 = 10/30 * |
| *Citrobacter* spp. | 5.9 = 2/34 | 11.1 = 4/36 | 5.1 = 2/39 | 20 = 6/30 |
| *Enterobacter* spp. | 5.9 = 2/34 | 13.9 = 5/36 | 15.4 = 6/39 | 6.7 = 2/30 |
| *Proteus* spp. | 5.9 = 2/34 | 2.8 = 1/36 | 0.0 = 0/39 ** | 0.0 = 0/30 |
| *Morganella* spp. | 2.9 = 1/34 | 0.0 = 0/36 ** | 0.0 = 0/39 ** | 0.0 = 0/30 |
| *Serratia* spp. | 0.0 = 0/34 ** | 5.5 = 2/36 | 2.6 = 1/39 | 0.0 = 0/30 |
| *Pantoea* spp. | 0.0 = 0/34 ** | 2.8 = 1/36 | 0.0 = 0/39 ** | 0.0 = 0/30 |
| *No Enterobacteriaceae* | | | | |
| *Aeromonas* | 0.0 | 100 = 1/1 | 0.0 | 0.0 |
| NFGNB | | | | |
| *Pseudomonas* spp. | 42.9 = 30/70 * | 51.6 = 33/64 * | 45.9 = 28/61 * | 50.0 = 33/66 * |
| *Stenotrophomonas* spp. | 17.1 = 12/70 * | 15.6 = 10/64 | 18 = 11/61 * | 27.3 = 18/66 * |
| *Alcaligenes* spp. | 10 = 7/70 | 9.4 = 6/64 | 6.6 = 4/61 | 3.0 = 2/66 |
| *Acinetobacter* spp. | 8.6 = 6/70 | 6.2 = 4/64 | 6.6 = 4/61 | 6.1 = 4/66 |
| *Achromobacter* spp. | 7.1 = 5/70 | 3.1 = 2/64 | 6.6 = 4/61 | 4.5 = 3/66 |
| *Sphingobacterium* spp. | 1.4% = 1/70 ** | 0% = 0/64 ** | 0.0 = 0/61 ** | 0.0 = 0/66 ** |
| *Brevundimonas* spp. | 1.4 = 1/70 ** | 1.6 = 1/64 ** | 0.0 = 0/61 ** | 4.5 = 3/66 |
| *Delftia* spp. | 1.4 = 1/70 ** | 1.6 = 1/64 ** | 1.6 = 1/61** | 0.0 = 0/66 ** |
| *Elizabethkingia* spp. | 1.4 = 1/70 ** | 0.0 = 0/64 ** | 1.6 = 1/61** | 1.5 = 1/66** |
| *Sphingomonas* spp. | 0.0 = 0/70 ** | 3.1 = 2/64 | 1.6 = 1/61** | 0.0 = 0/66 ** |
| GNBNI | 8.6 = 6/70 | 7.8 = 5/64 | 11.5 = 7/61 | 3.0 = 2/66 |
| *Fungus* | | | | |
| *Candida* | 0.0 = 0/113 | 1.8 = 2/110 | 0.8 = 1/118 | 2.8 = 3/108 |

FB, fermenting bacteria; NFGNB, Non-fermenting Gram-negative bacilli; GNBNI, Gram-negative bacilli not identified; * = species with a significantly high rate of occurrence;** = species with a remarkably low rate of occurrence.

Group F: 49.1%, p < 0.001), and *Klebsiella* spp. (Group M: 33.3%, p < 0.001; Group F: 29.1%, p = 0.001), while *Proteus* spp. (Group M: 2.4%, p = 0.006; Group F: 1.8%, p = 0.015), *Morganella* spp. (Group M: 0.0%, p < 0.001; Group F: 1.8%, p = 0.015), *Serratia* spp. (Group M: 3.6%, p = 0.014; Group F: 0.0%, p = 0.005), and *Pantoea* spp. (Group M: 1.2%, p = 0.002; Group F: 0.0%, p = 0.005) were the less represented bacteria.

Also among NFGNB, the most frequent strains were *Pseudomonas* spp. (Group M: 45.7%, p < 0.001; Group F: 50.0%, p < 0.001) and *Stenotrophomonas* spp. (Group M: 20.3%, p = 0.001; Group F: 18.5%, p = 0.003), while the less frequent strains were *Sphingobacterium* spp. (Group M: 0.0%, p < 0.001; Group F: 0.9%, p = 0.003), *Sphingomonas* spp. (Group M: 0.6%, p < 0.001; Group F: 1.8%, p = 0.007), *Brevundimonas* spp. (Group M: 2.0%, p = 0.002; Group F: 1.8%, p = 0.007), *Delphia* spp. (Group M: 0.0%, p < 0.001; Group F: 0.9%, p = 0.007), and *Elizabethkingia* spp. (Group M: 1.3%, p < 0.001; Group F: 0.9%, p = 0.003). About the comparison between Groups M and F, no significant differences among different strains were observed.

The 449 bile samples across age classes is shown in Table 4. Analyses into age groups analysis showed that among GPB, the *Enterococcus* spp. was the strain most significantly present in all groups apart from Group B (Group A: 88.9%, p = 0.002; Group C: 88.2%, p < 0.001; Group D: 100%, p = 0.002). Among GNB, the highest rate was for *E. coli* (Group A: 47.1%, p < 0.001; Group B: 38.9%, p < 0.001; Group C: 41.0%, p < 0.001; Group D: 40%, p < 0.001), and *Klebsiella* spp. (Group A: 32.3%, p < 0.001; Group C: 35.9%, p < 0.001; Group D: 33.3%, p = 0.003). Among NFGNB, the highest rate was for *Pseudomonas* spp. (Group A: 42.9%, p < 0.001; Group B:51.6%, p < 0.001; Group C: 45.9%, p < 0.001; Group D: 50%, p < 0.001), and *Stenotrophomonas* spp. (Group A: 17.1%, p = 0.044; Group C: 18.0%, p = 0.028; Group D: 27.3%, p < 0.001); while *Sphingobacterium* (Group A: 1.4%, p = 0.020; Group B: 0.0%, p = 0.008; Group C: 0.0%, p = 0.011; Group D: 0.0%, p = 0.006), *Brevundimonas* (Group A: 1.4%, p = 0.020; Group B: 1.6%, p = 0.026; Group C: 0.0%, p = 0.011), *Delftia* (Group A: 1.4%, p = 0.020; Group B: 1.6%, p = 0.026; Group C: 1.6%, p = 0.035; Group D: 0.0%, p = 0.006), *Elizabethkingia* (Group A: 1.4%, p = 0.020; Group B: 0.0%, p = 0.008; Group C: 1.6%, p = 0.035; Group D: 1.5%, p = 0.020), and *Sphingomonas* (Group A: 0.0%, p = 0.006; Group C: 1.6%, p = 0.035; Group D: 0.0%, p = 0.006), were the less represented bacteria. *Sphingobacterium* spp., *Delftia*, and *Elizabethkingia* were present with the lowest frequency in all categories. Concerning the GNB, the microbiological species analysis showed *K. pneumoniae* sequence (data not shown).

The inter-assay statistical analysis between the different groups by age did not show any significant difference in rates of microbiological species s. The death rate was 81.98%. The follow-up analysis of patients by groups showed that 30.23% of patients died in the ED group, 21.52% in the non-ED-1, and 23.26% in the non-ED-2 group. No significant difference was found between groups of survival (ED groups). No issues of missing data were encountered in our study, and all anonymous data are available for any researcher.

## Discussion

We aimed to identify the bile microbiota of Italian inpatients who underwent ERCP procedures using standard microbiological methods and robust statistical tools. The results have been correlated with the age and gender of the patients. The authors analyzed the positive bile isolates in four subgroups by age quartile values. Our study had a homogeneous numeric distribution of enrolled surgical patients. According to Italian demographic indicators, the aging index showed that, during the study period, the population aged 65 and over in our district rose from 17.5 to 18.2% (https://www.istat.it/en/sicilia/data). Our study shows both in univariate and multivariate analysis the prevalence of Gram-negative bacteria in all groups. In particular, in the elderly, in fact, in group D, in subjects > 80 years, the percentage of Gram-negative was 89%. These findings concur with previous studies that see older individuals at risk of modifying the intestinal microbiome with loss of permeability and the possibility of determining dysbiosis [22–24].

This data seems confirmed by the findings of the low rate of *Sphingobacterium* spp., *Delftia* spp., and *Elizabethkingia* spp. by aging. It affirms that the aging progression opens the doors to the potential implication in health and age-related disease [32,33].

The two typical indications, biliary obstruction caused by either bile duct stones or malignancy, accounted for more than 90% of all procedures performed in our series and increased indeed with age [6,9]. In our study, *Enterococcus* spp. was the prevalent strain in GPB genera. The frequency of *Enterococcus* spp. was statistically significant in all patients except for patients with age into the range of 67−74 years. Commonly, enterococci are relatively minor constituents of the human gastrointestinal microbiota (less than 1%). Still, the influence of the host's characteristics (age, diet, health status, and antibiotic treatment) gives a reason for the diversity and population frequency of different bacterial groups [18]. Other studies identified *Enterococcus* spp. as the most common bile isolate in a subject with bacteremic biliary tract infection [34]. Despite the epidemiological analysis on prevalent species, *Enterococcus* genus showed a low prevalence of vancomycin-related antibiotic resistance in Southern European regions [35]. Moreover, other species are emerging as pathogens in long-term-care facilities and hospital-acquired infections [36] with individual reports of *E. gallinarum* in our geographical area [37].

Therefore, the frequency of *Enterococcus* spp. in our series should be a red flag, mainly if the bile sample originates from the elderly. In this age group, comorbidities are often present. The insertion of a stent by ERCP for palliative treatment of an obstructed bile duct is responsible for the highest rate of *Enterococcus* spp. in cancer patients [38].

In the past decade, the literature stressed the critical role of *Streptococcus* spp., which should be considered when choosing antibiotics for therapy and prevention of biliary septicemia, mainly because of these bacteria's cardiovascular involvement sepsis [8,39,40]. In our study, *Streptococcus* spp. has been isolated at the lowest rate, especially in male patients. *Streptococcus* spp. is common in investigations that include patients with biliary gallstones [41]. Among GNB, we found a prevalence of *E. coli, Klebsiella* spp., *Pseudomonas* spp., and *Stenotrophomonas* spp. These pathogens are typically reported in studies conducted in patients with biliary tract infections and often have the same pathogens isolated from blood [42]. In our study, the data of bacteria, including multidrug-resistant (MDR) pathogens, such as *Pseudomonas* spp. or *Stenotrophomonas* spp., underlines how close we are to a hospital pathogenic flora.

The epidemiology of bacteria isolated from bile samples with positive culture has changed in recent years. Although some microorganisms, such as *E. coli* and *Klebsiella* spp., have been described in the last decade, their resistance to antibiotics has recently changed, especially in the Mediterranean area [28,43]. Our group described the outbreak of *K. pneumoniae* and *Acinetobacter baumannii* in surgery and intensive care units in Sicily, Italy [28,30,43]. In our patients, GNB was most isolated from female patients than males. This data may be interpreted considering that elderly females suffer more from urinary tract infections than age-related men. The urinary tract is indeed the most common primary source of initial gram-negative disease [44]. Recently, studies suggest that the intestinal barrier is composed of several integrated components that are physical, immunological, and microbiological evolves across the entire life [9–11]. Apart from the indiscriminate use of antibiotics as antibiotic prophylaxis in surgery that leads to a change in the flora of surgical patients, it is known that immunocompromised patients have a sparse gut microbiome repertoire, which may promote colonization by MDR bacteria [45]. Among uncommon bacteria, we described *Sphingobacterium* spp. and *Sphingomonas* spp. Both microorganisms have been reported as constituents of the biliary microbiome in a subgroup of subjects with gallstone disease and a typical Mediterranean diet composed of *caciocavallo* cheese, a type of stretched-curd cheese made from sheep's or cow's milk and produced throughout Southern Italy [46]. The role of compounds in most fruits and vegetables that showed anti-inflammatory activity in gut microbiota metabolism may be a critical factor in studying the influence of the alimentation on bile [47].

Although original in the conception and robust in the MATLAB-supported analysis of the microorganisms, our study has some limitations. It is a cross-sectional study and does not include lifestyle and epigenetic data that may be confounding factors. Our team opted not to add bile from cadavers because postmortem bacteremia is a relatively common phenomenon, with up to 76% of patients harboring contaminants at the time of the autopsy [48].

Finally, the bactibilia pattern described in our study may reflect the emergence and selection of some strains such as *K. pneumoniae* and *E. coli*, which have been responsible for hospital outbreaks in Southern Europe as reported from us and others [3,18,28,30,31,36,37,43,47,49,50]. We plan to explore modes of microbial transmission and gene frequency in several scenarios employing a Bayesian approach for predicting routes of contamination, revealing critical control points for microbial management. We hope that elucidating several communities' genetic landscapes and hospital-based environments may pose vital practical implications for healthcare providers and pharmaceutical companies.

In conclusion, our study emphasizes that bile isolates from young patients may have GPB, while old patients show GNB. GPB is more often found in males than in females. Future studies may encourage more extensive survey studies, including outpatients and the inclusion of risk factors such as diet, social habits, oncological disease, and stage, as well as sexual orientation.

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## Competing interests

None declared.

## Ethics committee approval

The Ethics Committee of the University of Palermo (Institutional Review Board, IRB) approved this study (Resolution Nr. UIN3220).

## Patient consent to publish

Written informed permission to conduct this research and publish this research was obtained from the patients. The consent can be requested at any time from the study coordinator (PDC).

## Availability of data and materials

Data are available from the corresponding author and/or study coordinator (PDC) on request.

## Author contribution

NS and PdC wrote the first draft of the manuscript. PdC performed the experiments, NS performed the statistical analysis and interpretation of data, FdA, EB, and TF performed the endoscopy, GG provided surgical data for some patients who underwent surgery, AG, VR and CM reviewed the first draft, CS reviewed the experimental data, the statistical analysis, and reviewed the last draft of the manuscript. All authors approved the final draft of the manuscript.

## Declarations

The study complies with the checklist of STROBE guidelines covering professional reports of cross-sectional studies. The authors declare no competing interests. Data in anonymous form and materials with details of procedures will be available at any time by the authors on the reviewers, journal, and academic authorities' request. All relevant raw (anonymous) data will be freely available to any scientist wishing to use them for non-commercial purposes, without breaching participant confidentiality. Internal funding from the University of Palermo has been provided for this study. Dr. C. Sergi has received grants from Women and Children's Health Research Institute, Canada, the Canadian Foundation for Women's Health, and the Canadian Institutes of Health Research.

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