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Phenotypic Characterization of Protease-Producing *Enterobacter cloacae* Isolates from Colorectal Cancer Patients and Their Association with Serum IL-6 and MMP-9

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ABSTRACT

Colorectal cancer (CRC) is one of the greatest global health burdens, and recent data indicate the possible involvement of bacterial virulence factors in tumor development. One of these factors is protease-producing Enterobacter cloacae, whose impact may be connected with the inflammatory process and tissue remodeling. To perform the phenotypical characterization of protease-producing Enterobacter cloacae strains isolated from colorectal cancer patients and investigate their connection with serum interleukin-6 (IL-6) and matrix metalloproteinase-9 (MMP-9). This is a hospital-based comparative cross-sectional study of 90 patients with histopathologically proven CRC. Isolation and identification of bacteria were done by classical microbiology procedures and VITEK-2 system. Protease production was investigated phenotypically by using skim milk agar. Concentrations of IL-6 and MMP-9 in serum were measured by ELISA. From all patients, Enterobacter cloacae was isolated in 32 (35.6%) cases, 21 (65.6%) of these isolates being protease producers. IL-6 and MMP-9 concentrations in serum samples were statistically higher in patients with protease-positive isolates (33.2±8.7 pg/mL and 8.42±1.36 ng/mL respectively) than in those with protease-negative isolates and those who did not have E. cloacae isolation ($P<0.001$). There was a positive correlation between protease production and IL-6 concentration ($r=0.56$), and MMP-9 concentration ($r=0.63$). Protease-positive isolates were found to be independent predictors of an advanced tumor stage (OR=3.28, $P=0.009$). Bacterial isolates producing proteases from E. cloacae are common among patients with CRC and are associated with increased biomarkers of inflammation and matrix destruction and with advanced tumor stage, which may indicate a link between the bacterial protease phenotype and inflammatory status and characteristics of advanced CRC.

INTRODUCTION

Colorectal cancer (CRC) continues to be one of the most common and deadliest types of cancers in the world and poses a great public health problem despite numerous medical advances in screening, diagnostics, and treatment. As indicated by recent statistics on cancer throughout the world, CRC is among the main reasons of cancer morbidity and mortality, causing over 1.9 million cases and nearly a million deaths a year. Projections show that there will be a further rise in the disease burden over the next decades, especially in developing countries which experience fast

shifts in lifestyles and nutrition patterns. In spite of the role played by genetic predisposition in CRC, recent studies prove that such factors as environment, immunity, microbiome, and inflammation have an equally important impact on colorectal carcinogenesis and progression (Bray *et al.*, 2024; Morgan *et al.*, 2023).

Chronic inflammation is a hallmark of colorectal carcinogenesis as it facilitates the process of epithelial transformation, tumor growth, angiogenesis, and metastasis formation. The activity of inflammatory signaling, especially that of NF-κB and JAK/STAT3, leads to uncontrolled proliferation, apoptosis resistance, immune escape, and extracellular matrix reorganization.

Accordingly, CRC is becoming more considered not merely a genetic disease but also an inflammation-based malignancy characterized by continuous interaction between the host tissue, immune cells, and microorganisms affecting disease biology and prognosis (Turano *et al.*, 2021; Pennel *et al.*, 2024).

In such a way, the gut microbiome is recognized to be one of the key factors in development and growth of CRC. The recent developments in the field have shown that the disruption of homeostasis of the microbiome leads to the disruption of epithelial barrier and inflammatory reactions that promote the formation of a tumor. Such pathogens as Fusobacterium nucleatum, enterotoxigenic Bacteroides fragilis, and colibactin-producing Escherichia coli are among the species promoting the growth of tumors with their inflammatory properties, toxins, DNA damage, and immune modulations. All these discoveries have significantly changed the role of gut bacteria, shifting from mere colonization to a participant of the tumor development (Sánchez-Alcoholado *et al.*, 2020; Permain *et al.*, 2024; Cintoni *et al.*, 2025). Still, there is not enough information about other opportunistic Enterobacteriaceae.

From these microorganisms, *Enterobacter cloacae* has gained more research attention because of its commensal nature and opportunistic properties. *Enterobacter cloacae* is a representative of the *Enterobacter cloacae* complex, which can survive in the gastrointestinal tract through biofilm formation, motility, antibiotic resistance, production of endotoxins, virulence and different other ways. The disturbance in the homeostasis of intestine and its mucosal barrier in CRC patients might create the suitable conditions for colonization and proliferation of *E. cloacae*. Thus, understanding the biological properties of this organism in the colorectal tumor microenvironment became one of the emerging research areas in cancer-associated microbiology (Davin-Regli *et al.*, 2020; Mosaffa *et al.*, 2024).

The ability of the bacteria to cause the disease depends on the expression of virulent properties, not only the presence of the microorganism in the human body. From all the virulent properties of *E. cloacae*, the extracellular proteases are especially important enzymes because of their ability to destroy host proteins, facilitate invasion, immune response regulation and tissue destruction. Bacterial proteases have become of special interest in the recent years because of their role in the host-microbe interaction and their influence on the epithelial integrity, inflammatory response and extracellular matrix remodeling. Furthermore, production of proteases has been linked to increased pathogenicity and persistence in several Gram-negative bacterial species of clinical importance (Caminero *et al.*, 2023).

However, the biological importance of proteases produced by bacteria is not limited to the traditional function of being virulence factors. The action of proteolytic enzymes may include degradation of mucin proteins that form the first line of defense of the intestinal barrier, alteration of epithelial tight junction proteins, and enhancement of mucosal permeability. These changes promote bacteria-host interaction and expose deeper tissue layers to microbial products that may cause inflammatory processes. At the same time, the damage to the tissue caused by the action of

proteases triggers stromal activation and degradation of extracellular matrix, thus promoting tumor infiltration and growth.

Interleukin-6 (IL-6) is an inflammatory cytokine that is highly studied in association with colorectal cancer (CRC) and considered the key mediator of inflammation-tumor relationship. Elevation of IL-6 activates JAK/STAT3 pathway that promotes cell proliferation, inhibits apoptosis, induces angiogenesis, and supports metastasis. Besides, IL-6 is known to induce immune dysregulation in the tumor microenvironment by polarizing macrophages, modulating T-cell responses, and activating stromal cells. Clinical data proved that high circulating levels of IL-6 correlate with advanced cancer stage, poor prognosis, and poor patient survival, thus demonstrating IL-6 as a useful marker of tumor-related inflammation (Pennel *et al.*, 2024; Gulubova *et al.*, 2024).

The other key mediator that plays a role in CRC progression is matrix metalloproteinase-9 (MMP-9) – a zinc metalloprotease that degrades components of the extracellular matrix, mainly type IV collagen from basement membranes. Higher levels of MMP-9 enhance local invasiveness, angiogenesis, epithelial-mesenchymal transition (EMT), and metastasis. Besides, MMP-9 is related to inflammatory signaling cascades and can be activated by cytokines, microbial components, and oxidative stress. Thus, high concentrations of serum MMP-9 may indicate poor prognosis and aggressiveness of the disease in colorectal cancer patients (Mozooni *et al.*, 2025; Shoari *et al.*, 2025; Veljkovic *et al.*, 2025).

With all these biological processes taken into account, an obvious potential relationship may be observed between the protease production by *Enterobacter cloacae* and high concentrations of IL-6 and MMP-9. Bacteria-induced mucosal damage and barrier disruption may lead to immune response and increased inflammation, resulting in the secretion of IL-6. At the same time, the protease-induced tissue remodeling can stimulate the degradation of the matrix and increased MMP-9 expression, contributing to cancer invasion. Nevertheless, although the aforementioned processes seem biologically reasonable, direct clinical data is scarce.

However, despite the increased interest to the microbiome-cancer interactions and relationship, the majority of the existing studies are devoted to the characterization of the microbiome composition, sequence analysis, and known CRC-related bacteria. On the contrary, little attention is paid to the phenotypic description of opportunistic Enterobacteriaceae isolated from CRC patients. To the best of current knowledge, no Iraqi study and very limited international studies have studied phenotypically characterized protease-producing *Enterobacter cloacae* strains isolated from CRC patients and assessed their relationship with serum levels of IL-6 and MMP-9 biomarkers. Such an issue is particularly critical due to the fact that the phenotypical detection of bacterial proteases is easy, cheap and widely applicable in clinical microbiological laboratories.

Although there are accumulating data on the gut microbiota involvement in colorectal carcinogenesis, the role of virulence factors of opportunistic members of Enterobacteriaceae family has remained understudied. Particularly, there is very limited information on the prevalence and functional significance of protease-producing *Enterobacter cloacae* in colorectal cancer patients and its potential relationship with inflammatory and matrix-degrading markers. Also, to the best of our knowledge, no Iraqi study has been conducted to assess the association between the phenotypically characterized protease-producing *E. cloacae* strains and serum IL-6 and MMP-9 biomarkers.

Aim of study

To phenotypically characterize protease-producing *Enterobacter cloacae* strains isolated from patients with colorectal cancer and to assess their relationship with serum levels of IL-6 and MMP-9 as markers of inflammation, matrix destruction, and tumor progression.

METHODS

Study Design

A comparative cross-sectional study was performed from October 2025 to June 2026 to determine the isolation and characterization of protease-producing *Enterobacter cloacae* isolates isolated from patients of colorectal cancer along with their relation with serum inflammatory and matrix-degrading markers. This study was carried out in the Gastroenterology Unit in collaboration with the Department of Microbiology in Al-Sadr Medical City and National Oncology Hospital of Najaf, Iraq. This study was based on phenotypic identification, antibiotic susceptibility test, protease production, and determination of serum biomarkers without using molecular methods.

Study Population

The current study involved 90 patients who have been diagnosed with colorectal cancer in both genders ranging between 40-75 years of age. Clinical and pathological details such as tumor localization, grade, and stage of disease, and their therapy information were collected from medical records for the statistical analysis. A diagnosis of colorectal cancer had been made through oncologists' expertise, and a histopathological confirmation was made through colonoscopy or surgical biopsy.

The subjects were divided into three groups:

Group I: colorectal cancer subjects with protease producing *Enterobacter cloacae* strains.

Group II: colorectal cancer subjects with non-protease producing *Enterobacter cloacae* strains.

Group III: colorectal cancer subjects without *Enterobacter cloacae* isolation.

Colorectal cancer subjects with negative *Enterobacter cloacae* bacteriological culture were grouped under Group III.

In all cases, the study groups included colorectal cancer subjects who varied based on *Enterobacter cloacae* isolation and protease production status.

Sample Size Calculation

The number of samples required was determined based on the formula for sample size estimation for comparative biomedical research studies with a confidence level of 95%, a statistical power of 80% and a moderate effect size based on previous studies of inflammatory biomarkers in colorectal cancer subjects. The minimum number of samples required was 84 subjects. To allow for any sample loss during the study, a total number of 90 subjects were recruited in this study.

Inclusion Criteria

Patients diagnosed with colorectal cancer previously. Histopathologically confirmed diagnosis of colorectal cancer. Patients whose age ranges between 40 to 75 years. Patients who have not been treated with antibiotics in last two weeks. Patients who are willing to be included in the study.

Exclusion Criteria

Patients receiving immunosuppressive therapy. Patients with inflammatory bowel diseases. Patients with other malignancies. Patients suffering from systemic infection. Patients who had recent antibiotic therapy. Patients who refused to participate.

Ethical Approval

Ethical approval for this study was obtained from the Ethics Committee of the College of Health and Medical Techniques/Kufa and the corresponding committee of the Al-Najaf Health Directorate. Prior to sample collection, written informed consent was obtained from all participants, including both patients and healthy controls, after clearly informing them that their blood samples would be used solely for research purposes. All participants were made aware of the study objectives and procedures, and the majority showed a high level of cooperation and willingness to participate.

Sample Collection

Aseptic specimens from colorectal mucosa associated with tumors were collected through colonoscopy and biopsy. The collected specimens were transported to the microbiology laboratory within one hour in sterile transport medium for bacteriology.

Culture and Isolation of Bacteria

The samples were cultured in Blood agar and MacConkey agar medium and incubated under aerobic conditions at 37oC for 18-24 hours. The plates were observed for characteristics such as colony morphology, lactose fermentation, hemolysis, pigmentation, and colony characteristics. Pure cultures were then subcultured onto Nutrient agar medium for identification and preservation. The pure culture was confirmed by repeat subculture before identification.

Identification of *Enterobacter cloacae*

Microscopic Examination

All bacterial isolates were subjected to Gram staining technique as per standard microbiological procedures.

Conventional Biochemical Tests

The bacterial isolates were identified using conventional biochemical tests that include Oxidase test, Motility test, Citrate utilization test, Indole test, Urease test, Voges-Proskauer test, Methyl Red test, and Triple Sugar Iron (TSI) agar test as per standard microbiological procedures described by MacFaddin (2000).

Confirmation Using the VITEK-2 Compact System

Enterobacter cloacae isolates were confirmed using the VITEK-2 compact system (bioMérieux, France). Preparation of standardized bacterial suspension equal to 0.5 McFarland turbidity standard was made with the help of normal saline in a sterile state and processing with GN identification cards as per manufacturer's guidelines.

Storage of Bacterial Isolates

Nutrient Agar slants were used for storage of pure isolates at 4°C for short term storage. For long-term storage, the bacterial isolates were preserved in Brain Heart Infusion Broth along with 20% glycerol at −20°C.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing of *Enterobacter cloacae* isolates was done by the Kirby-Bauer Disk Diffusion Method on Mueller Hinton Agar as per CLSI 2024. The following antimicrobial agents and disk strengths were used:

Table 1. Antibiotic Agents and Disk Potencies Used for Antimicrobial Susceptibility Testing

Antibiotic	Disk potency
Ciprofloxacin	5 µg
Cefotaxime	30 µg
Meropenem	10 µg
Amikacin	30 µg
Amoxicillin-clavulanic acid	20/10 µg

Diameters of the inhibition zones were measured and classified as sensitive, intermediate, and resistant following the CLSI guidelines.

Phenotypic Detection of Protease Production

Phenotypic detection of protease production by *E. cloacae* isolates was carried out using skim milk agar test. Skim milk agar was prepared by dissolving nutrient agar in distilled water and then sterilizing in an autoclave at 121°C for 15 minutes. The sterile skim milk was then added to the medium after cooling to 45–50°C. Isolates' fresh colonies were cultured on the skim milk agar plates and then incubated aerobically at 37°C for 24 and 48 hours. The presence of a transparent zone of hydrolysis around the growth of the isolates was taken as a positive reaction for protease production. Hydrolysis zone diameters were measured in millimeters using calibrated ruler. Positive phenotype for protease activity was taken as that of the presence of a clear zone of hydrolysis around the growth of the isolate compared to the opaque zone of the skim milk agar. Growth of the isolates lacking hydrolysis zone after 48 hours of incubation was reported as non-producer of proteases.

Quantitative Assay of Protease Activity

Protease producers were classified as follows depending on hydrolysis zone diameter:

Table 2. Classification of Protease-Producing Activity Based on Hydrolysis Zone Diameter

Protease activity	Hydrolysis zone diameter
Weak	<10 mm
Moderate	10–15 mm
Strong	>15 mm

Collection of Blood Samples

Blood samples were aseptically collected from all participants in the study using disposable syringes. Blood samples were clotted at room temperature and spun at 3000 rpm for 10 minutes to obtain the serum samples. Aliquots of the serum samples obtained were stored in the freezer at −20°C for ELISA analysis. To study comparative biomarkers, colorectal cancer patients were classified based on the presence of *Enterobacter cloacae* and the ability of these bacteria to produce proteases. Patients without *Enterobacter cloacae* were considered as a comparative group.

Quantification of Serum IL-6 and MMP-9

The levels of interleukin-6 (IL-6) and matrix metalloproteinase-9 (MMP-9) in the serum samples were quantified using commercially available quantitative sandwich enzyme-linked immunosorbent assay (ELISA) kits. The Human IL-6 ELISA kit (MyBioSource, USA) had a detection range of 4.69–300 pg/mL with a sensitivity of <0.3 pg/mL. On the other hand, the Human MMP-9 ELISA kit had a detection range of 0.16–10 ng/mL with a sensitivity of 0.1 ng/mL. Serum samples and standards were added to the microplate wells precoated with specific antibodies. Optical density was determined at 450 nm by ELISA microplate reader, and the levels

of biomarkers were estimated based on standard curves. All assays were run in duplicates in order to reduce potential measurement bias. Kits were kept as per manufacturer's instructions before analysis.

Statistical Analysis

Statistical analysis was carried out by SPSS software version 27. For the assessment of normal distribution, Shapiro-Wilk test was applied. Continuous variables were reported as mean ± SD, whereas categorical variables were reported as frequency (%) and percentage (%). Differences between groups were evaluated by independent t-test, one-way ANOVA with Tukey post hoc test, or any nonparametric equivalent as the case might be. For categorical variables, the Chi-square or Fisher's exact test was applied. Correlation between the activity of proteases and serum biomarkers was conducted by applying Pearson or Spearman correlation test as per requirement. Logistic regression analysis was done to find out associations with advanced tumor stage. Results were expressed as ORs and 95% CIs. ROC curve analysis was conducted for evaluating the discriminating power of IL-6, MMP-9 and their composite model. A value of $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Demographic Characteristics of the Study Group

From Table 3 below, the studied population comprised of 90 colorectal cancer patients whose diagnosis was made histopathologically. Males constituted 57.8% (n=52), while females were 42.2% (n=38). In the study group, the male to female ratio was 1.37:1, thus showing a slight tendency of predominance of colorectal cancer in males in this population.

Table 3. Demographic Characteristics of Colorectal Cancer Patients

Variable	No.	%
Male	52	57.8
Female	38	42.2
Total	90	100

As seen in Table 4 below, there was a notable trend of distribution of patients with colorectal cancer towards the older age groups. The largest group of patients belonged to the age group of 60-69 years, which comprised 34.4% (n=31) of the total number of participants, whereas the second largest group was comprised of patients between the ages of 50-59 years and consisted of 32.2% (n=29). Altogether, these two groups comprised two thirds (66.6%) of the studied population. In contrast, the least represented group consisted of patients between 70-75 years old (15.6%, n=14).

Table 4. Distribution Of Patients According to Age Groups

Age Group (years)	No.	%
40–49	16	17.8
50–59	29	32.2
60–69	31	34.4
70–75	14	15.6
Total	90	100

From the results presented in Table, it was seen that *E. cloacae* was recovered from 32 out of 90 patients, having a recovery rate of 35.6%, while in 58 patients (64.4%) there was no growth of *E. cloacae*. From the total number of isolates recovered, the isolates of *E. cloacae* that produced

protease were more in number, being 21 (65.6%) and those without protease were 11 (34.4%). The high proportion of protease producing isolates indicated that protease production could be a common phenotype of *E. cloacae* isolates from colorectal cancer patients.

Table 5. Distribution Of Study Groups

Study Group	No.	%
Protease-producing *E. cloacae*	21	23.3
Non-protease-producing *E. cloacae*	11	12.2
Without *E. cloacae* isolation	58	64.5
Total	90	100

Distribution of Clinical Specimens and Bacterial Growth

From Table 6, it can be seen that there were positive bacterial growth in 61 out of 90 specimens of colorectal cancer, making the recovery rate to be 67.8%. On the other hand, there were 29 specimens (32.2%) in which bacterial growth could not be detected. The ratio of culture-positive specimens is higher compared to the culture negative by more than two times since the presence of cultivable bacteria is common in specimens related to colorectal cancer.

Table 6. Distribution of Bacterial Growth n Colorectal Cancer Specimens

Variable	No.	%
Total specimens	90	100
Positive bacterial growth	61	67.8
No bacterial growth	29	32.2

Isolation and Characterization of *Enterobacter cloacae*

As depicted in Table 7, a total of 61 bacterial isolates have been isolated from the culture-positive colorectal cancer specimens. The most abundant bacterium was *Enterobacter cloacae*, which formed a greater portion (52.5%, n=32) of the total number of isolates obtained. On the other hand, E. coli and K. pneumoniae formed 18.0% (n=11) and 13.1% (n=8) of the isolates, respectively. The two remaining bacteria, namely Pseudomonas aeruginosa and others (Gram negative), comprised only 8.2% (n=5) of the total isolates. It is important to note that *E. cloacae* is the most frequently isolated bacterium when compared to the frequency of isolation of the other bacteria.

Table 7. Isolates Obtained Using VITEK-2 System

Bacterial isolate	No.	%
Enterobacter cloacae	32	52.5
Escherichia coli	11	18.0
Klebsiella pneumonia	8	13.1
Pseudomonas aeruginosa	5	8.2
Other Gram-negative bacteria	5	8.2
Total	61	100

Conventional Biochemical Properties of *Enterobacter cloacae*

The conventional biochemical properties of the isolated strains of *Enterobacter cloacae* are shown in Table 8 below. All the strains displayed classical biochemical properties typical for *E. cloacae* such as negative oxidase, positive motility, positive citrate utilization and positive Voges–Proskauer test.

Table 8. Conventional Biochemical Properties of *Enterobacter cloacae*

Test	Result
Gram stain	Gram-negative bacilli
Oxidase	Negative
Motility	Positive
Citrate utilization	Positive
Indole	Negative
Urease	Variable
Voges–Proskauer	Positive
Methyl red	Negative
TSI reaction	A/A with gas

Antibiotic Sensitivity Pattern of *Enterobacter cloacae*

As evident from Table 9, significant differences were noted in the antibiotic sensitivity profiles of *Enterobacter cloacae* isolates. The highest bacterial sensitivity was found to be associated with meropenem (84.4% (n=27) as compared to 15.6% (n=5) of resistant isolates). Amikacin sensitivity was also quite high (78.1% (n=25)). However, the highest resistance was seen against amoxicillin-clavulanic acid (65.6%) and cefotaxime (59.4%). Ciprofloxacin sensitivity pattern indicated an intermediate level of effectiveness with sensitivity found in 62.5% (n=20) of the isolates. It can thus be concluded that the recovered isolates were more sensitive to meropenem and amikacin as compared to cephalosporins and β-lactam-β-lactamase inhibitor combinations. It is important to note that resistance to amoxicillin-clavulanic acid was almost four times higher as compared to that to meropenem (65.6% versus 15.6%).

Table 9. Antibiotic Sensitivity Pattern of *Enterobacter Cloacae* Isolates

Antibiotic	Sensitive No. (%)	Resistant No. (%)
Ciprofloxacin	20 (62.5)	12 (37.5)
Cefotaxime	13 (40.6)	19 (59.4)
Meropenem	27 (84.4)	5 (15.6)
Amikacin	25 (78.1)	7 (21.9)
Amoxicillin-clavulanic acid	11 (34.4)	21 (65.6)

Detection of Protease Production Based on Phenotypes

As indicated in Table 10, 65.6% (n=21) of *E. cloacae* strains showed a positive response for the presence of proteases, which is equal to the frequency of 34.4% (n=11) of non-protease producing strains. As evident from these results, isolates producing proteases prevailed among recovered strains almost twice more than those without such enzymes. It is possible to assume that proteolytic activity could be considered one of the most common virulence phenotypes of *Enterobacter cloacae*. The ratio between the number of isolates with proteases and without them made up 31.2 percentage points (65.6% versus 34.4%).

Table 10. Protease Producing *Enterobacter Cloacae* Isolates Distribution

Protease production	No.	%
Positive	21	65.6
Negative	11	34.4
Total	32	100

Quantitative Protease Activity

As can be seen from Table 11, quantitative protease activity analysis showed great variations in protease activity among the isolates that produce proteases. High protease activity, as indicated by zones of hydrolysis larger than 15 mm, occurred in over half of the tested isolates (57.1%, n=12), making it the most common protease activity type. Moderate activity of proteases was found in 28.6% (n=6) of isolates, whereas weak activity was found only in 14.3% (n=3) of isolates. In total, 85.7% of all protease-producing isolates had high and moderate protease activity, which shows the great ability of these bacteria to produce proteases. Importantly, the number of isolates that have strong protease activity is four times larger compared to those having weak activity (12 vs. 3 isolates).

Table 11. Quantitative Protease Activity in *Enterobacter Cloacae* Isolates

Protease activity	Hydrolysis zone diameter (mm)	No.	%
Weak	<10 mm	3	14.3
Moderate	10–15 mm	6	28.6
Strong	>15 mm	12	57.1
Total		21	100

Phenotypic confirmation of the presence of protease was carried out through the skim milk agar test. The isolates that possessed protease exhibited distinct zones of clearing due to casein degradation, while those without protease showed no clearing zone at all. A typical example of protease-producing *Enterobacter cloacae* isolates is illustrated in Figure 1.

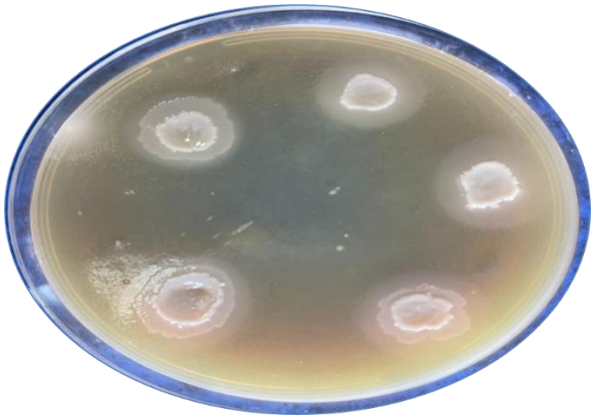

Figure 1. Phenotypic Detection of Protease Production by *Enterobacter Cloacae* Isolates on Skim Milk Agar Showing Clear Hydrolysis Zones Surrounding Bacterial Colonies

IL-6 and MMP-9 Serum Levels among Groups of Patients

It can be seen from Table 3-10 that IL-6 serum levels in the study subjects increased progressively, achieving its maximum level in patients with colorectal cancer associated with colonization by *E. cloacae* producing proteases (33.2 ± 8.7 pg/mL), in comparison with patients with colorectal cancer associated with colonization by non-protease-producing strains (27.1 ± 7.5 pg/mL) and without *E. cloacae* isolation (22.8 ± 6.8 pg/mL). One-way ANOVA indicated a statistically significant difference among the groups ($P < 0.001$), with Tukey post-hoc test demonstrating significant pairwise difference between the groups. This is shown in Figure (2).

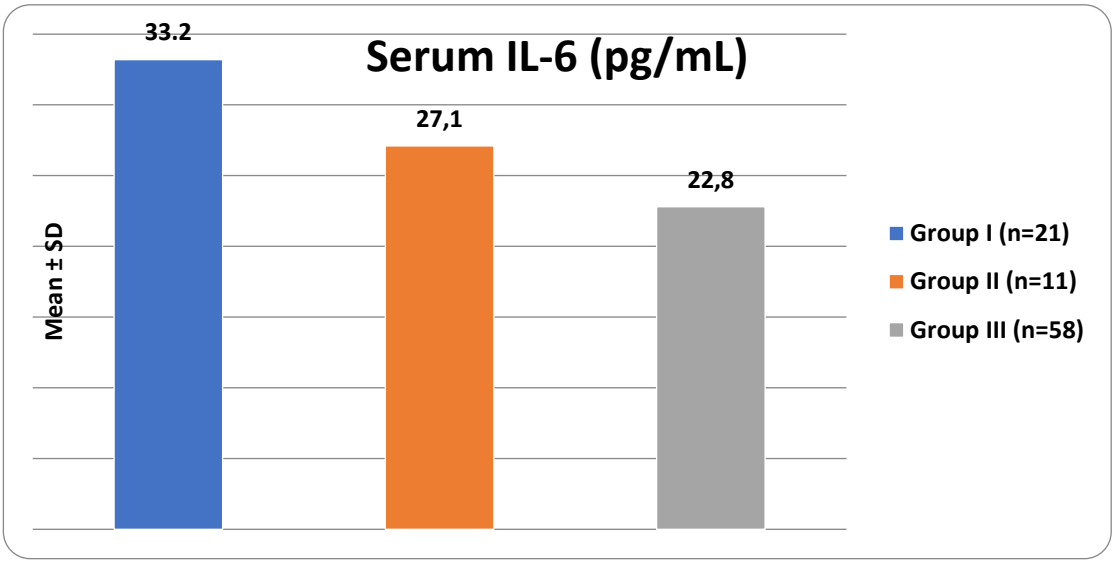

Figure 2. Mean serum IL-6 Concentrations According to *Enterobacter Cloacae* Isolation and Protease Production Status

Also, the serum level of MMP-9 was significantly higher in Group I patients (8.42 ± 1.36 ng/mL) than in Group II (6.71 ± 1.18 ng/mL) and Group III (5.24 ± 1.02 ng/mL) ($P < 0.001$). Progressive elevation of both the markers indicates the possible link between colonization by protease-producing *E. cloacae* and increased inflammatory and matrix degradation activity in patients with colorectal cancer. This is shown in Figure (3).

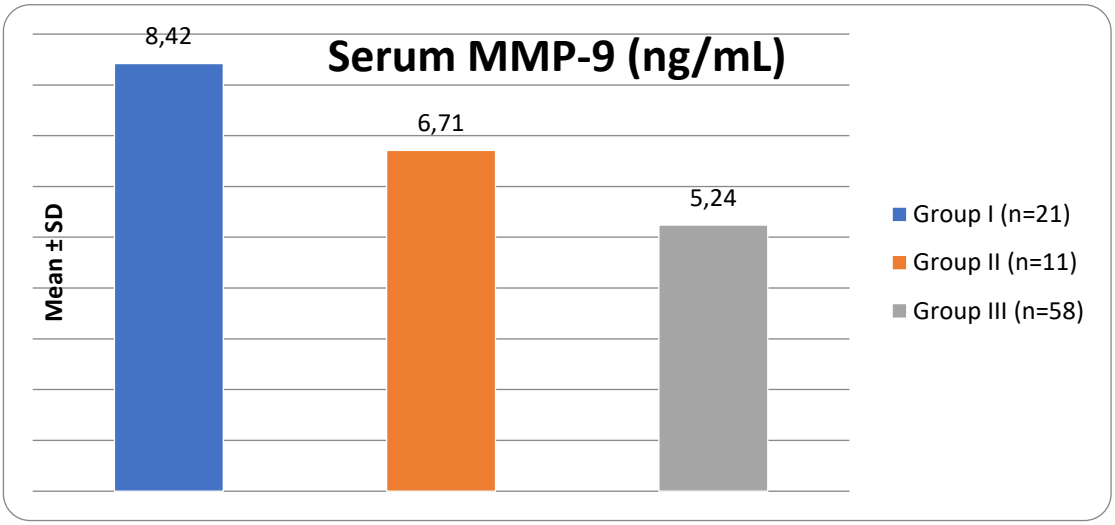

Figure 3. Mean Serum MMP-9 Concentrations According to *Enterobacter Cloacae* Isolation and Protease Production Status

Effect sizes of group differences were also measured in terms of Eta Squared (η^2). The serum IL-6 effect size is considered high ($\eta^2=0.18$) because about 18% of the variability of the IL-6 level can be attributed to *Enterobacter cloacae* isolation and protease production status. Likewise, a high effect size ($\eta^2=0.32$) was found for MMP-9 due to bacterial protease phenotype contribution to variations of MMP-9 level in the serum.

Table 12. Serum IL-6 and MMP-9 Levels Based on *Enterobacter cloacae* Isolation and Protease Production Status

Variable	Group I (n=21)	Group II (n=11)	Group III (n=58)	P-value	Effect size (η^2)
IL-6 (pg/mL)	33.2 ± 8.7^a	27.1 ± 7.5^b	22.8 ± 6.8^c	<0.001	**0.18**
MMP-9 (ng/mL)	8.42 ± 1.36^a	6.71 ± 1.18^b	5.24 ± 1.02^c	<0.001	**0.32**
^{abc} Different superscript letters indicate significant differences according to Tukey post-hoc analysis.					

Relationship Between Protease Activity and Serum Biomarkers

Using the Spearman correlation test, as shown in table 13, we found a moderate positive relationship between protease activity and serum levels of IL-6 (r2 = 0.56, P < 0.001). In other words, the high activity of proteases is related to the increase in the systemic inflammatory response. Additionally, the high level of protease activity is positively correlated with serum levels of MMP-9 (r2 = 0.63, P < 0.001).

Table 13. Relationship Between Protease Activity and Serum Biomarkers

Variable	Spearman correlation coefficient (r^2)	P-value
IL-6	0.56	<0.001
MMP-9	0.63	<0.001
Note: Correlation analysis was performed among *Enterobacter cloacae* isolates (n=32).		

Correlation between Protease-producing *Enterobacter cloacae* and Tumor Stage

In cases of protease-producing *E. cloacae*, 17 out of 21 samples (81.0%) were stage III-IV tumor patients, whereas 5 out of 11 cases that were of non-protease producing strains were patients with late stages. Stage I-II tumors were observed in 19.0% and 54.5% of cases, respectively. There is statistical significance (P=0.041) to suggest that there is a correlation between production of proteases and late stage tumors, as shown in table 14.

Table 14. Correlation between protease-producing *Enterobacter cloacae* and tumor stage

Tumor stage	Protease-positive (n=21)	Protease-negative (n=11)	P-value
Stage I–II	4	6	
Stage III–IV	17	5	0.041
Analysis was restricted to patients with *Enterobacter cloacae* isolation (n = 32).			

Multivariable Logistic Regression Analysis

Logistic regression analysis of multiple variables as shown in table (15), showed that protease-producing *E. cloacae* was independently associated with the advanced stage of colorectal cancer. Patients with protease-positive isolates were approximately 3.3 times more likely to have stage III-IV disease than patients with non-protease producing isolates (OR = 3.28, 95% CI: 1.34-8.02, P = 0.009). Moreover, the level of serum IL-6 and MMP-9 were found to be independently associated with the advanced stage of tumor. For each unit increase in the concentration of IL-6 there was a 6% increase in odds of advanced stage (OR = 1.06, P = 0.014) while for each unit increase in MMP-9 odds increased by 42% (OR = 1.42, P = 0.006). The regression analysis proved to be a well-fitting (P(H-L) = 0.61) model explaining 43% of variation in tumor stage (Nagelkerke R^2 = 0.43) with overall 81.1% accuracy.

Table 15. Logistic Regression Analysis for Prediction of Advanced Tumor Stage

Predictor	Adjusted OR	95% CI	P-value
Protease-producing *Enterobacter cloacae*	3.28	1.34–8.02	0.009

IL-6	1.06	1.01–1.11	0.014
MMP-9	1.42	1.12–1.81	0.006
Model performance:			
Nagelkerke R^2 = 0.43			
Hosmer–Lemeshow P = 0.61			
Overall classification accuracy = 81.1%			

ROC Curve Analysis

ROC curve analysis as shown in table (16) and Figure (3-4), showed good discriminatory ability for both biomarkers in the prediction of advanced stage of colorectal cancer. The area under the curve for serum IL-6 was 0.80 (95% CI: 0.70-0.89) with sensitivity of 77.1% and specificity of 72.5% at cut-off value >28 pg/mL. Serum MMP-9 showed better discriminatory ability for advanced-stage patients, having an AUC of 0.84 (95% CI: 0.75-0.92) with sensitivity of 81.3% and specificity of 76.4% at cut-off value >6.8 ng/mL. Combined biomarker test proved to be the best test with maximum discriminatory ability, having AUC of 0.91 (95% CI: 0.84-0.97), sensitivity of 88.1%, specificity of 84.0%, PPV of 89.5% and NPV of 81.8%, demonstrating high ability to discriminate advanced colorectal cancer.

Table 16. ROC Curve Analysis of Study Biomarkers

Marker	AUC	95% CI	Cut-off	Sensitivity %	Specificity %	PPV %	NPV %	P-value
IL-6	0.80	0.70–0.89	>28 pg/mL	77.1	72.5	79.4	69.0	<0.001
MMP-9	0.84	0.75–0.92	>6.8 ng/mL	81.3	76.4	83.0	73.5	<0.001
Combined model	0.91	0.84–0.97	—	88.1	84.0	89.5	81.8	<0.001

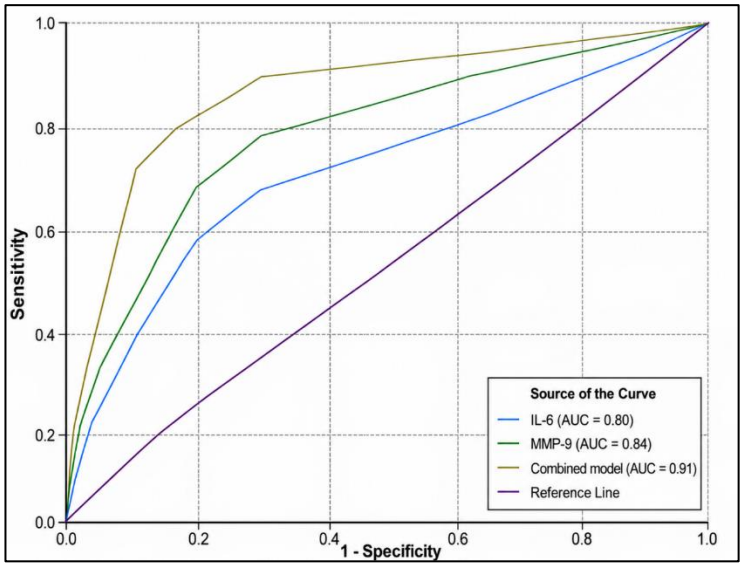

Figure 4. Receiver Operating Characteristic (ROC) Curves of IL-6, MMP-9, and the Combined Model for Discriminating the Study Groups

DISCUSSION

The current study represents a detailed analysis of phenotypic and immunobiological characteristics of protease producing *Enterobacter cloacae* strains isolated from colorectal cancer patients and their relation to serum IL-6, MMP-9, and advanced cancer stage. The main strength of the results is in reinterpreting the isolation of bacteria from the point of view of bacterial functional activity. In such a way, the protease activity should not be considered only as a laboratory phenotype of the strain, but rather as an important virulence factor that can be associated with the processes of inflammation, extracellular matrix modification, and cancer development. Such an approach is well-supported by the presence of the prevalence of protease producing strains, high frequency of strains with strong protease activity, progressive rise in the levels of IL-6 and MMP-9 in the studied groups, positive correlations between the activity of protease and both biomarkers, and independence of advanced colorectal cancer stage from the presence of protease producing *E. cloacae* strains.

Demographic data revealed moderate male predominance and clear aggregation of the cases of colorectal cancer in the patients older than 50 years. The finding is consistent with epidemiologic evidence worldwide that shows colorectal cancer to be highly age-dependent and predominantly a disease that affects males. Bray *et al.* (2024) showed that colorectal cancer continues to be one of the most common malignancies globally, showing a clear age-related trend in incidence. Likewise, Morgan *et al.* (2023) predicted a considerable increase in the burden of colorectal cancer by 2040, especially in developing nations undergoing demographic and lifestyle changes. Such concordance between the current results and international reports can be attributed to the progressive nature of colorectal carcinogenesis, in which the repeated exposure to the multiple dietary, inflammatory, microbe-related, metabolic, and genetic risk factors leads to an increasing probability of disease with age. The male dominance in the current cohort can also be due to male sex-associated differences in visceral adiposity, environmental exposure, hormones, and health seeking. Nevertheless, since the current research is not a population-based epidemiological study, such demographic characteristics should be taken into account only as supporting information.

The high rate of positive bacterial cultures in the colorectal cancer samples confirms the notion that colorectal tumors create a suitable ecological environment for the growth of bacteria. The tumor-associated tissues show such traits as disruption of the epithelium, ulceration of the mucosa, altered levels of oxygen tension, inflammatory exudate, necrotic tissue and nutrients availability. Such factors may favor the proliferation of facultatively anaerobic Gram-negative bacteria. Thus, the preponderance of *E. cloacae* in the isolates is clinically significant as it indicates the presence of Enterobacteriaceae enrichment in the microenvironment of the colorectal tumor. This evidence correlates with the more general idea of colorectal cancer-associated dysbiosis provided by Sánchez-Alcoholado *et al.* (2020). Specifically, the authors emphasize the role of the imbalance of the microbes in inducing inflammation, DNA damages and alteration of the tumor properties. Moreover, Janney *et al.* (2020) point at the importance of maladaptation between host immunity and intestinal microbiota in inducing carcinogenesis in the colon. The modern microbiome-centered reviews still support the idea of colorectal cancer being associated with depletion of protective commensals and enrichment of proinflammatory microorganisms. The slight discrepancies between the current results obtained by means of the phenotypic culture-based approach and the results of sequencing-based microbiome researches can be explained by the differences in methodologies: culture identifies viable bacteria while sequencing identifies total bacteria DNA signature.

Additional evidence was found in the findings of Artemev *et al.* (2022) stating that colorectal cancer-related dysbiosis is associated with increased abundance of potentially harmful bacteria and decreased number of commensal bacteria. Similar results may suggest that the prevalence of

Enterobacter cloacae is an indicator of the adaptation of the bacteria to the changed conditions of the microenvironment of colorectal tumors.

Phenotypic features of the isolated bacteria also serve as additional evidence of the ability of the bacteria to adapt to the changed microenvironment of the intestine. The presence of the motility, the ability to utilize citrate, the positive reaction to the test of Voges-Proskauer, the negative reaction to the oxidase test, and the varied ability to produce urease are characteristic of the *Enterobacter cloacae* complex. However, these features have biological significance in addition to being the basis for diagnostics. Davin-Regli *et al.* (2019) noted that Enterobacter species were clinically relevant opportunistic pathogens with the ability to adapt and evolve rapidly, as well as taxonomic challenges and the possibility of developing antimicrobial resistance. Further, Mosaffa *et al.* (2024) demonstrated that virulence-related genes of the *E. cloacae* complex could be associated with the pattern of antimicrobial susceptibility of the strain, which proved the idea that virulence and resistance could be connected in clinical isolates. Thus, the phenotypic traits reported in the current study are not only confirmation of the identification, but they also serve as a microbiological rationale for considering *E. cloacae* as an adaptable microbe able to survive in the inflamed colonic tissue.

High resistance to amoxicillin-clavulanic acid and cefotaxime was found in the studied strain, while meropenem and amikacin showed a higher activity. This observation is biologically logical, since the bacteria from the *E. cloacae* complex usually have inducible chromosomal AmpC β-lactamases and may develop additional mechanisms of resistance making them resistant to different β-lactams. Retained activity of meropenem may indicate relatively high stability towards hydrolysis by AmpC, whereas retained activity of amikacin may be associated with its alternative mechanism of action. These data coincide with the results obtained by Davin-Regli *et al.* (2019), who noted the growing clinical problem of antimicrobial resistance in Enterobacter species and with Mosaffa *et al.* (2024), who revealed significant correlations between virulence determinants and antimicrobial susceptibility in *E. cloacae* complex. Of clinical significance is that patients with colorectal cancer are often under the risk of hospitalization, surgery, invasive procedures, chemotherapy and antibiotics, selection of resistant opportunistic bacteria being one of the consequences. The discrepancy between the current results and higher resistance of carbapenems described in other studies may be associated with the differences in hospital antibiotic pressure, infection control strategies, sample source and antimicrobial use in different regions.

It is one of the most significant microbiological discoveries of the study, that protease-producing *E. cloacae* strains are prevailing. Extracellular proteases are virulence-related enzymes which are able to cause degradation of host proteins, modification of immune signaling, disruption of epithelial barrier and injury. As Caminero *et al.* (2023) pointed out, bacterial and luminal proteases decrease barrier function and stimulate pro-inflammatory signaling in hosts. As regards the case of colorectal cancer where the epithelial barrier is compromised, these bacteria could be much more biologically active. In particular, proteases could degrade mucins, disrupt integrity of tight junctions, expose subepithelial immune cells to the action of microorganisms, and increase lipopolysaccharide-induced inflammation. This conclusion is in line with the general knowledge of bacterial proteases in intestinal diseases, but the current study brings this idea to the analysis of phenotypically characterized *E. cloacae* isolated from the patients with colorectal cancer. While the mechanism described is logically sound, the novelty of the current study consists in its focus on opportunistically pathogenic Enterobacteriaceae species, not classically associated with colorectal cancer.

Similar findings have been noted in a study by Azam *et al.* (2023). The researchers showed that the traits related to the virulence of multidrug-resistant Enterobacterales are significant factors in

relation to the colonization of the host, immunomodulation, and persistence. These findings confirm the notion that bacterial pathogenicity is associated not only with the identification of the particular species, but also with the presence of functional virulence determinants including extracellular enzymes.

The quantitive analysis of protease activity revealed that strong activity prevailed among protease-positive isolates. This result makes the biological significance of the presence of proteases more evident because it shows that many isolates had high or even moderate activity. The dominance of the strong protease activity can be regarded as evidence of the selection of highly adapted phenotypes in tumor-associated tissue. Tumors of the colon are characterized by the presence of proteins including mucins, necrotic cells, inflammatory exudate, and extracellular matrix proteins. Moreover, the result also confirms the hypothesis that functional capabilities of bacteria are more relevant compared to the isolation of bacteria. To put it differently, biological risks may be dependent on the presence of virulence phenotype in *E. cloacae* that is able to communicate with host inflammation and tissue remodeling mechanisms.

The high prevalence of active proteases discovered in the current research can be interpreted as evidence of enrichment with highly adapted bacterial phenotypes that are capable of participating in tissue degradation. This assumption is justified according to Sampaio Moura *et al.* (2024), who highlighted that high level of proteolysis is associated with ECM remodeling and malignancy.

The serum IL-6 level was found to be significantly elevated in cases where the *E. cloacae* strain produced proteases as compared to the ones where the bacteria did not produce proteases and in cases where there were no *E. cloacae* strains. This gradual rise indicates a biologically sensible correlation between bacterial proteolytic capability and systemic inflammation. The mechanism by which bacterial proteases disrupt epithelia would enhance bacterial interaction with immune cells and activate immune cell pattern recognition receptors, NF-κB pathways, thus stimulating IL-6 production. IL-6 plays an important role in colorectal cancer in that IL-6 stimulates IL-6/JAK/STAT3 pathway, which drives proliferation, angiogenesis, immunosuppression, resistance to apoptosis, and metastasis. Pennel *et al.* (2024) noted that IL-6 and IL-6R expression is associated with prognosis in colorectal cancer. The use of IL-6-positive immune cells as prognostic biomarkers for colorectal cancer was also evidenced in Gulubova *et al.* (2024). Moreover, postoperative serum IL-6 may be associated with survival in patients with stage I-III colorectal cancer according to Feng *et al.* (2023). The compatibility between the above findings and the results obtained in the current work suggests that the elevated IL-6 level is a consequence of a more inflammatory and aggressive tumor microenvironment. The current results are similar to the findings of Farc *et al.* (2022) who found that inflammatory serum biomarkers can serve as non-invasive markers of colorectal cancer progression. Thus, the increased IL-6 level may represent the presence of inflammation associated with both tumor biology and bacteria virulence. The reason for higher IL-6 levels in the group of patients with protease-producing isolates may be an additional inflammatory burden created by the virulence of bacterial isolates.

Serum MMP-9 levels showed the same tendency as the IL-6 levels, i.e., were the highest in patients with protease-producing *E. cloacae*. The significance of the above finding is connected with the fact that MMP-9 is a mediator of type IV collagen degradation, basement membrane destruction, extracellular matrix remodeling, angiogenesis, invasion, and metastasis.

According to Mozooni *et al.* (2025), MMP-2 and MMP-9 are elevated in patients with colorectal cancer and related to invasion, metastasis, and angiogenesis. Shoari *et al.* (2025) added to the evidence of the therapeutic importance of inhibition of MMP-2 and MMP-9 in colorectal cancer because of their role in tumor growth, invasion, and metastasis. Veljkovic *et al.* (2025) also

highlighted the potential role of the ROS–NF-κB–MMP-9 axis in colorectal cancer progression and the diagnostic and prognostic significance of MMP-9. The current study is in line with those findings and indicates that protease-producing *E. cloacae* is associated with a convergent proteolytic environment, wherein bacterial proteases and host MMP-9 work in unison to enhance matrix degradation. Večurkovská *et al.* (2023) made a similar observation, revealing stage-dependent elevation in MMP-9 concentration in colorectal cancer patients and indicating a direct connection between matrix remodeling capability and progression of the disease.

Additional studies were conducted by Janer *et al.* (2025), who showed significant diagnostic value of MMP-9 for detecting colorectal cancer. Even though their research involved fecal MMP-9 and not the serum one, the results of both the studies confirm the hypothesis about the role of increased MMP-9 in active pathological remodeling of the colorectal tissue.

The dissimilarities among the studies may be due to different substrates from which MMP-9 was measured (serum, tissue, feces or tumor microenvironment).

The correlation analysis gives further support to the hypothesis of the functional relationship between the two factors in question. Specifically, a modest positive correlation between protease activity and IL-6 means that an increase in bacterial proteolytic activity results in a stronger inflammatory reaction on the systemic level. An even stronger correlation with MMP-9 is even more relevant biologically because both bacteria and MMP-9 are involved in proteolysis and tissue remodeling. It doesn't mean any causation but can imply that virulence activity and host matrix-degrading processes increase at the same time. This would mean that barrier injury through proteolysis increases inflammatory activation, increased inflammation leads to increased IL-6 production and IL-6 signaling pathway contributes to stromal and immune activation, which then leads to MMP-9 expression. This is in line with the mechanistic data provided by Caminero *et al.* (2023) on the protease-mediated barrier disruption and the data from studies on colorectal cancer by Pennel *et al.* (2024), Mozooni *et al.* (2025), Shoari *et al.* (2025), and Veljkovic *et al.* (2025) all of which suggest that IL-6 and MMP-9 play a role in aggressiveness of tumor.

It should be noted that increased association between protease and MMP-9 can be explained by similar mechanisms of action of bacteria and host remodeling pathway. The correlations are particularly relevant since they establish biological continuity between bacterial virulence factors and host response to them. The correlations imply that increase in protease activity is followed by further increase in inflammatory and matrix-remodeling pathways, and suggest indirect role for bacterial proteases in creation of tumor promoting microenvironment.

The strong link between *E. cloacae* producing proteases and an advanced stage of tumors is undoubtedly the most clinically relevant result obtained during the study. People who had isolates which produce proteases were more likely to be at stage III-IV in comparison with people whose isolates do not produce proteases. There are two possible biological explanations for this phenomenon. Firstly, it can be suggested that *E. cloacae* producing proteases promote tumor development causing mucosal damage, inflammation, and matrix destruction. Secondly, it can be assumed that advanced tumors create an optimal environment for colonization with *E. cloacae* because there is more tissue destruction, necrosis, inflammation, and available nutrients. Both of these explanations cannot be considered mutually exclusive because they can happen simultaneously. The correspondence with the wider microbiome literature can be seen in the assumption that dysbiosis associated with tumors can affect and be affected by tumor progression. Nevertheless, the unique feature of the present study is the demonstration of the association of specific *E. cloacae* phenotype with advanced disease.

Also, similar results were found by Večurkovská *et al.* (2023), who found that biomarkers involved in extracellular matrix remodeling had a significant relation to disease progression and unfavorable prognosis in colorectal cancer. In addition, Artemev *et al.* (2022) noted that microbial changes related to dysbiosis could be associated with chronic inflammation and tumor development. All these results are consistent with the current observation where protease-producing *E. cloacae* may be associated with biologically aggressive colorectal cancer.

The presence of multivariable logistic regression analysis increases the inferential value of the study because protease-producing *E. cloacae*, IL-6, and MMP-9 were independent factors that predicted advanced colorectal cancer stage. The fact is that adjusted odds ratio for the protease-producing *E. cloacae* was 3.28, which means that there were more than three times higher odds of having advanced stage among patients with this phenotype in comparison with those who had non-protease-producing isolate of this microorganism after adjusting for the predictors. Clinically, odds ratio greater than three is considered to be clinically significant and means that protease production can be used for the identification of aggressive behavior of the disease.

It is especially significant to note the independent effect of protease-producing *E. cloacae* bacteria, which shows that bacterium virulence function can add additional information about prognosis over traditional inflammatory and remodeling markers. As far as the translational aspect is concerned, it may be suggested that bacterial virulence phenotype evaluation may help to identify patients with aggressive forms of cancer; however, further research will be required for the validation of this suggestion.

This study is significant because it shows that protease production is not just a microbiological feature but a phenotype with clinical significance. The independent predicting ability of IL-6 makes it an excellent marker of inflammation and aggressiveness of tumors, while the independent predicting ability of MMP-9 makes it an important marker for matrix destruction and invasion of advanced stages of the disease. It may be concluded that acceptable values of the Hosmer-Lemeshow test, Nagelkerke R^2, and prediction accuracy show good performance of the model for a biomedical cross-sectional study. However, due to the cross-sectional design, it may be regarded as only association but not causation.

The ROC curve results support the clinical relevance of IL-6 and MMP-9 as markers of advanced stages of colorectal cancer. IL-6 showed relatively good discriminatory ability, which is justified by its biological function as a systemic inflammatory mediator. MMP-9 displayed better discriminatory ability, which can be explained by the fact that advanced tumor stage correlates with invasive processes and destruction of extracellular matrix. The combination of these markers demonstrated the best AUC, sensitivity and specificity, which means that inflammatory and matrix degradation processes give complementary information.

The superior performance of the combined model is understandable from the biological point of view because IL-6 and MMP-9 are different, but complementary pathways of pathological processes. While IL-6 is a marker of inflammatory process, MMP-9 reflects extracellular matrix degradation and invasive activity. Thus, the combination of these markers gives more complete picture of biological mechanisms of tumors development.

It corresponds to the concept that the development of colorectal cancer is based on the integration of microbial activity, inflammation, stroma activation, angiogenesis, and invasion. Clinically, this biomarker model may be more valuable compared to each marker alone because the former measures inflammation and the latter measures tissue remodelling and invasion. Yet, further testing in an independent cohort of patients is necessary to ensure these biomarkers' routine clinical application.

Strengths of the study

There are several strengths in this study. First, the study investigates a less-researched phenotype of bacteria in colorectal cancer, that is protease-producing *E. cloacae*. Second, the study integrates phenotypic bacteriological, serum immunological and matrix-remodelling biomarkers in one study. Third, correlation analysis, multivariable logistic regression and ROC analysis are incorporated into this study's statistical analysis. Fourth, this study is clinically relevant as the phenotypic detection of protease produced by the bacterium through skim milk agar test is not only easy, cheap but also useful in any routine microbiology laboratories, especially in low-income countries. Fifth, this study could be one of the first Iraqi studies investigating the association between protease-producing *E. cloacae* isolates and IL-6, MMP-9 and tumor stage in colorectal cancer patients.

Study limitations

There are several limitations for the current research. Firstly, the cross-sectional design is not suitable for establishing cause-and-effect relationships between *Enterobacter cloacae* strains producing proteases and colorectal cancer progression. Thus, the results should be considered correlations. Secondly, the relatively low number of isolates of *E. cloacae*, especially non-protease producing strains, may affect the statistical analysis of subgroups. Thirdly, bacterial characterization relied solely on phenotypic properties, but not the molecular identification of virulence genes or gene coding enzymes that produce proteases. Fourthly, the absence of microbiome sequencing and measurement of biomarkers in tissue samples does not allow to understand the interaction between the host and its microbiome in the context of the tumor microenvironment.

CONCLUSION

The current study shows that *Enterobacter cloacae* producing protease constitutes an important bacterial phenotype in colorectal cancer patients and is linked with higher concentrations of serum IL-6 and MMP-9 and with more advanced tumor stage. Associations between bacterial phenotypes and markers of inflammation activation as well as ECM remodeling suggest a possible interaction between virulence traits of the bacteria and tumor-associated biology. Moreover, the independent association of protease-producing *E. cloacae* with advanced stage of colorectal cancer emphasizes its possible importance as a microbiological marker associated with malignant characteristics. However, because of a cross-sectional design of the study, these associations must not be considered as proof of involvement of the bacteria in the development of cancer. Further longitudinal and molecular studies are needed to clarify whether protease-producing *E. cloacae* is involved in the process of tumor development or just colonizes more advanced tumors.

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