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Microbiological risk assessment and resistome analysis from shotgun metagenomics of bovine colostrum microbiome

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ABSTRACT

Colostrum is known for its nutraceutical qualities, probiotic attributes, and health benefits. The aim of this study was to profile colostrum microbiome from bovine in rural sites of a developing country. The focus was on microbiological safety assessments and antimicrobial resistance, taking into account the risks linked with the consumption of raw colostrum. Shotgun sequencing was employed to analyze microbiome in raw buffalo and cow colostrum. Alpha and beta diversity analyses revealed increased inter and intra-variability within colostrum samples' microbiome from both livestock species. The colostrum microbiome was mainly comprised of bacteria, with over 90% abundance, whereas fungi and viruses were found in minor abundance. Known probiotic species, such as *Leuconostoc mesenteroides*, *Lactococcus lactis*, *Streptococcus thermophilus*, and *Lactobacillus paracasei*, were found in the colostrum samples. A relatively higher number of pathogenic and opportunistic pathogenic bacteria were identified in colostrum from both animals, including clinically significant bacteria like *Clostridium botulinum*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Listeria monocytogenes*. Binning retrieved 11 high-quality metagenome-assembled genomes (MAGs), with three MAGs potentially representing novel species from the genera *Psychrobacter* and *Pantoea*. Notably, 175 antimicrobial resistance genes (ARGs) and variants were detected, with 55 of them common to both buffalo and cow colostrum metagenomes. These ARGs confer resistance against aminoglycoside, fluoroquinolone, tetracycline, sulfonamide, and peptide antibiotics. In conclusion, this study describes a thorough overview of microbial communities in buffalo and cow colostrum samples. It emphasizes the importance of hygienic processing and pasteurization in minimizing the potential transmission of harmful microorganisms linked to the consumption of colostrum.

1. Introduction

Colostrum refers to the nutrient-rich first milk produced by the mammals' mammary glands during lactation. It is characterized by high levels of antibodies, essential nutrients (carbohydrates, fats, proteins, and omega-3 fatty acids), growth factors, and antioxidant properties, which support to establishing the immune system and growth of the newborn (Marnila and Korohnen, 2002; McGrath et al., 2016; Playford and Weiser, 2021). Nutraceutical features and health benefits have made colostrum an emerging food containing higher concentrations of several important biomolecules (beta-lactoglobulin, alpha-lactalbumin, and caseins) than milk (Marnila and Korohnen, 2002). The lactoferrin (1.5 g/L) in bovine colostrum is crucial for various protective and physiological features such as immunomodulatory activity, anticancer activity, bowel-based iron absorption, non-immune protection, and antimicrobial activities (Giansanti et al., 2016). The minerals and

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vitamins present in colostrum may act as antioxidants or facilitate the antioxidative process, serving to prevent the synthesis of reactive oxygen species (Ceniti et al., 2022; Moretti et al., 2020). Colostrum's potential probiotic properties are also being focused (Damaceno et al., 2017). The human consumption of animal colostrum does not affect calf welfare as colostrum production in healthy cows is naturally more than the needs of the calves (Ceniti et al., 2022). The colostrum quality evaluation is primarily associated with the quantification of nutrients and immunoglobulins. Moreover, acceptable standards include a total bacterial count below 100,000 cfu/mL and coliform counts of less than 10,000 cfu/mL (Godden et al., 2019). Previous studies warn against the consumption of raw milk products (Alegbeleye et al., 2018; Fagnani et al., 2021). Colostrum microbiome profiling and thorough microbiological safety assessments should also be integrated into the quality evaluation.

In recent years, there has been a significant surge in research focused on the microbiology of colostrum (Hang et al., 2020; Liu et al., 2020; Messman and Lemley, 2023). These studies revealed the presences of a diverse and dynamic microorganisms population in colostrum, which is referred to as colostrum microbiome and is considered crucial for the newborn's health and growth (Hang et al., 2020; Messman and Lemley, 2023). Colostrum microbiome (fungi, bacteria, and viruses) is known to perform a variety of functions such as immune system modulation, growth enhancement, development of gut microbiome, and protection against pathogens (Lima et al., 2017; Messman and Lemley, 2023). The entero-mammary pathway in mice and humans reported to facilitate bacterial movement from the gut to mammary glands and thus is considered a potential source of colostrum microbiota origin (Selvamani et al., 2021). The literature reveals that intramammary environment and milk favor a broad range of environmental bacteria (Chen et al., 2021; Drago et al., 2017). Various studies reported the presence of several bacterial phyla, including Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria, in colostrum (Chen et al., 2021; Hang et al., 2020). Commonly identified bacterial genera in colostrum comprise of *Ruminococcaceae*, *Pseudomonas*, *Enterococcus*, *Staphylococcus*, *Bacteroides*, *Lactobacillus*, and *Corynebacterium* (Hang et al., 2020; Messman and Lemley, 2023; Vasquez et al., 2022). However, a substantially varied microbiome distribution has been noticed in cattle milk and colostrum (Chen et al., 2021; Taponen et al., 2019).

Effective colostrum management is essential to obtain high-quality colostrum and ensure its microbial quality (Miranda et al., 2023). This is not only necessary for the calves' survival and health but also for the well-being of humans who consume colostrum and its derivatives. A study in the United States stated that 39 % of bovine colostrum contained the microbial population within acceptable limits such as $<$10,000 cfu/mL of total coliform counts and $<$100,000 cfu/mL of total plate counts whereas the other colostrum samples demonstrated bacterial infection risk to calves (Morrill et al., 2012). Similarly, colostrum examination in Czech dairy farms depicted heavy microbial contaminations through total coliform counts, non-coliform gram-negative bacteria counts, and total plate counts (Slosarkova et al., 2021). The study further revealed that colostrum isolated bacterial pathogens mainly originated from fecal and environmental contaminations, and also contained commensal animal skin and mucosal microbiota. The authors also detected *Streptococcus uberis*, *Enterococcus* spp., *Streptococcus dysgalactiae*, *Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus parauberis* pathogens in the colostrum samples (Slosarkova et al., 2021). Ingestion of bovine colostrum contaminated with pathogenic bacteria can adversely impact calf health, potentially resulting in higher mortalities. The consumption of raw colostrum (without thermal treatment) can be potentially harmful to humans (Alegbeleye et al., 2018), which could enhance the antibiotic resistance risk and facilitate its food chain-based dissemination among humans (Miranda et al., 2023). Moreover, a recent study has unveiled that a majority of *Enterococcus* spp. recovered from bovine colostrum demonstrated multidrug resistance (MDR). This suggests that colostrum contaminated with MDR bacteria could potentially serve as a reservoir and a means of transmission for these resistant strains (Cunha et al., 2021). Another study identified $bla_{\text{TEM-171}}$ and $bla_{\text{CTX-M-15}}$ genes in ESBL-producing *E. coli* carried in colostrum fed to dairy calves (He et al., 2021).

The domestic buffalo is a major contributor to global milk production and serves as the main milk-producing animal in Pakistan and India. The cattle and buffalo colostrum could differ in protein and fat content (Abd El-Fattah et al., 2012). Overall, studies on buffalo colostrum are comparatively scarce than goats, cows, and sheep (Chen et al., 2021; Niyazbekova et al., 2020; Vasquez et al., 2022). Moreover, most of the colostrum microbiome studies employed 16S amplicon sequencing to analyze bovine samples, which effectively reveals the colostrum microbiome taxonomy at the genus level (Hang et al., 2020; Niyazbekova et al., 2020). However, shotgun metagenomic sequencing can taxonomically identify the colostrum microbiome up to the species level and reveal the microbial community's functional potential. This study investigated the raw colostrum microbiome (cows and buffalos), which is commonly consumed in Pakistan where pasteurization awareness and facilities are limited, and people believe in the nutritional benefits of raw dairy products. Furthermore, colostrum samples were also examined for the presence of pathogens and associated antimicrobial resistance genes (ARGs), which had never been thoroughly investigated.

2. Materials and methods

In this study, ten bovine colostrum samples were collected from rural sites on small homemade dairy farms in the Potohar region of Pakistan. Raw colostrum samples, obtained within 48 h after parturition, were collected in quantities of five samples from each buffalo (BuC) and cow (CoC) from five different farms at the selected site. These samples were collected in clean, sterilized 15 ml tubes, transported in an icebox, and subsequently stored at -80 °C.

2.1. Genomic DNA extraction and shotgun sequencing

Genomic DNA from the collected colostrum samples was extracted using bead-beating method, as previously described (Yasir et al., 2023). Briefly, one ml of each sample was centrifuged at 13,000 g for 10 min to eliminate the fat layer. Subsequently, the top fatty layer and supernatant were removed, and the pellet was reconstituted in 1.5 ml of PBS using vortexing for 30 s. After another centrifugation at 13,000 g for 10 min, the supernatant was removed, and DNA was extracted from the resulting pellet using the DNeasy PowerSoil Pro Kit (Qiagen, Germany).

The concentration of extracted DNA was measured using the Qubit Fluorometer in conjunction with the Qubit dsDNA high-sensitivity kit (Invitrogen, USA). Subsequently, DNA libraries were created, featuring insert sizes of ~400 base pairs, employing the Nextera DNA Flex Library Preparation Kit (Illumina, Inc., USA). Quality assessment and quantification of each sample's prepared library was carried out using Agilent D1000 HS tapes on the TapeStation 4200 (Agilent Technologies, USA). The barcoded libraries were then combined in equimolar amounts, and sequencing was carried out using the 2 $\times$ 250 bp V2 kit on a MiSeq system with a 500-cycles kit (Yasir et al., 2020).

2.2. Metagenomic bioinformatics analyses

Raw reads were processed for quality control, involving the removal of barcodes, adapters, and low-quality reads with a quality score below 20. Metagenomic analysis was conducted using the KBase platform, as described previously (Arkin et al., 2018; Yasir et al., 2022). In brief, Barcodes were trimmed using Trimmomatic v0.36 (Bolger et al., 2014). The microbial community was examined from unassembled paired-end sequence reads utilizing the Kaiju v1.9.0 (Menzel et al., 2016). The metaSPAdes - v3.15.3 tool was employed to create metagenomic assemblies, while metagenome-assembled genomes (MAGs) were constructed with MaxBin2 (Nurk et al., 2017; Wu et al., 2016; Yasir et al.,

2022). The quality of MAGs was evaluated using the CheckM tool, and further analysis with GTDB-Tk programs determined their novelty and taxonomic identification (Chaumeil et al., 2022; Parks et al., 2015). MAGs were annotated using BV-BRC tools (Olson et al., 2023). A maximum likelihood-based phylogenetic analysis of the obtained MAGs was conducted employing the Species Tree v2.2.0 tool, incorporating closely related genomes sourced from the NCBI database for microbial genomes (Price et al., 2010). The phylogenetic tree was visualized using the Interactive Tree of Life web tool (Letunic and Bork, 2021). Venn diagrams were produced using the InteractiVenn tool (Heberle et al., 2015).

The distribution of pathogenic bacteria in colostrum microbiomes were assessed using the CZ ID pipeline (Kalantar et al., 2020). ARGs were detected from the sequence reads using ARGs-OAP v2.0 tool and were subsequently normalized to reads per kilobase per million (rpkm) (Yin et al., 2018). ARGs-OAP utilizes a database known as SARG, which integrates the Comprehensive Antibiotic Resistance Database (CARD) and the Antibiotic Resistance Genes Database (ARDB). Open reading frames sequences were extracted from the MAGs utilizing the Prodigal tool. The sequences were aligned with CARD 3.1.4 by employing Resistance Gene Identifier v5.2.0 tool and Diamond algorithm (Alcock et al., 2020; Buchfink et al., 2015). The identification of ARGs from the MAGs was conducted with stringent significance criteria, employing CARD-curated bitscore cut-offs.

2.3. Statistical analysis

Alpha and beta diversity analyses were carried out within the MicrobiomeAnalyst pipeline to uncover the distribution of bacterial communities among the studied samples (Lu et al., 2023). The significance of beta diversity among the colostrum samples from buffalo and cow was assessed using Principal Coordinates Analysis (PCoA) based on permutational MANOVA. The Wilcoxon rank sum test and Pearson correlation tests were applied to determine differences in abundance of taxa between cow and buffalo colostrum. Statistical analyses were executed using SPSS 22 software (IBM, USA).

3. Results

3.1. Bacterial diversity analysis

In alpha diversity, Chao 1, accounting for species richness, and observed species revealed no significant difference ($p > 0.05$) between buffalo and cow colostrum (Fig. 1A, B). Cow samples (17.2 ± 1.4) showed slightly higher Fisher's index values than buffalo (16.9 ± 7.1), indicating higher evenness in the cow samples, but this difference was statistically not significant ($p > 0.05$) (Fig. 1C). There was no significant difference ($p > 0.05$) in the Shannon diversity index between buffalo (2.0 ± 0.6) and cow (2.0 ± 0.1) samples (Fig. 1D). Intra-sample variability in alpha diversity was apparent in the buffalo colostrum samples (Fig. 1A–D). The sequencing depth was sufficient in this study to encompass the bacterial species richness of all analyzed colostrum samples, as indicated by rarefaction curves and Good's coverage index average (100.0 ± 0.0) (Fig. S1). The principal coordinate analysis plot showed that samples from buffalo and cow were dissimilar but broadly clustered together (Fig. 1E). The differences in bacterial structure between colostrum samples from the two animals were not significant (F-value = 2.2, R-squared = 0.2, and $p = 0.08$) using the Bray–Curtis index and the PERMANOVA statistical methods (Fig. 1E).

3.2. Bacterial community analysis

A total of 23 bacterial phyla were recovered in the microbiome analysis of buffalo and cow colostrum. In buffalo colostrum, Firmicutes and Proteobacteria were the dominant microbiota, cumulatively accounting for over 75 % relative abundance in each sample (Fig. S2). In cow colostrum, Proteobacteria were predominantly detected (83.6 % ± 31.6 %), followed by Firmicutes (16.2 % ± 31.6 %). Actinobacteria were found at a relatively high average proportion of 5.1 % in buffalo colostrum, whereas they were present at less than 0.1 % in cow colostrum samples (Fig. S2). In the core microbiome, seven families were identified as common to both colostrum types, comprised of Pseudomonadaceae, Clostridiaceae, Moraxellaceae, Yersiniaceae, Enterobacteriaceae, Enterococcaceae, and Erwiniaceae (Fig. S3A). Among these families, Pseudomonadaceae and Clostridiaceae were predominantly found in most of the colostrum samples (Fig. S3B). From pairwise comparison using the Wilcoxon rank sum test, 23 families were detected with significantly different relative abundances ($p < 0.05$) between the colostrum samples from buffalo and cow including Streptomycetaceae, Methylobacteriaceae, Chromatiaceae, Comamonadaceae, Moraxellaceae, Erwiniaceae, Rhodobacteraceae, and Staphylococcaceae (Fig. S3C).

In total, 410 genera were identified in the colostrum samples. Among them, 117 genera were common to both buffalo and cow colostrum, while 233 genera were specifically detected in buffalo, and 60 genera were unique to cow colostrum (Fig. 2A). In the core microbiome analysis, five core genera *Pseudomonas*, *Rahnella*, *Clostridium*, *Enterococcus*, and *Pantoea* were commonly identified in both colostrum types. On average, these genera were detected at relatively high abundance, except for *Enterococcus* and *Pantoea*, which were found at <1.0 % abundance in buffalo colostrum (Fig. 2B). From pairwise comparisons using the Wilcoxon rank sum test, 18 genera were identified with significantly different relative abundances between the two colostrum types, including *Paracoccus*, *Macrococcus*, *Arthrobacter*, *Corynebacterium*, *Microbacterium*, *Edwardsiella*, *Brochothrix*, *Nocardia*, *Sphingobium*, and *Streptomyces* (Fig. 2C).

In total, 1029 species were found in the colostrum samples. A relatively larger number of species (734) were identified in buffalo colostrum samples compared to cow colostrum samples (527) (Fig. 3A). Among the identified species, 232 species were common to both animal colostrum, while 502 species were unique to buffalo, and 295 were exclusively found in cow colostrum (Fig. 3A). In the percentage relative abundance analysis, 37 species were identified, each of which was present at a level of at least 1.0 % in at least one colostrum sample. In the core microbiome analysis, ten species were commonly found in both buffalo and cow colostrum. The core species primarily included various *Pseudomonas* species, *Clostridium botulinum*, *Rahnella* sp. ERMR1:05, and *Pantoea agglomerans* (Fig. 3B). These ten core species had a relative abundance greater than 0.01 %, and none of them demonstrated a significant difference in relative abundance between the colostrum of buffalo and cow ($p > 0.05$). Overall, the relatively dominant species varied among colostrum samples from both buffalo and cow. Notably, *C. botulinum* was predominantly found at >10 % relative abundance in most buffalo colostrum samples compared to cow colostrum (Fig. 3C). Gram-positive bacteria *Carnobacterium maltaromaticum* from the Lactobacillales order was predominantly found in two of the buffalo colostrum samples. *Macrococcus caseolyticus* from Staphylococcaceae was detected at >10 % abundance in BuC4. *Pseudomonas* species were predominantly found in most cow samples, with *Pseudomonas* sp. J380 being present at >50 % abundance in three samples (Fig. 3C).

3.3. Probiotic and pathogenic bacteria

In the colostrum samples, a total of 17 known probiotics were identified, with 13 from buffalo and 12 from cow. Among these, eight probiotic species *Leuconostoc mesenteroides*, *Enterococcus faecium*, *Lactococcus lactis*, *Lactococcus garvieae*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Streptococcus thermophilus*, and *Lactobacillus paracasei* were common to both colostrum types (Table S1). A relatively larger number of 80 pathogenic, opportunistic, and rare pathogenic bacteria were detected in both colostrum types. Among the pathogenic bacteria, several are clinically significant, such as *C. botulinum*, *Pseudomonas*

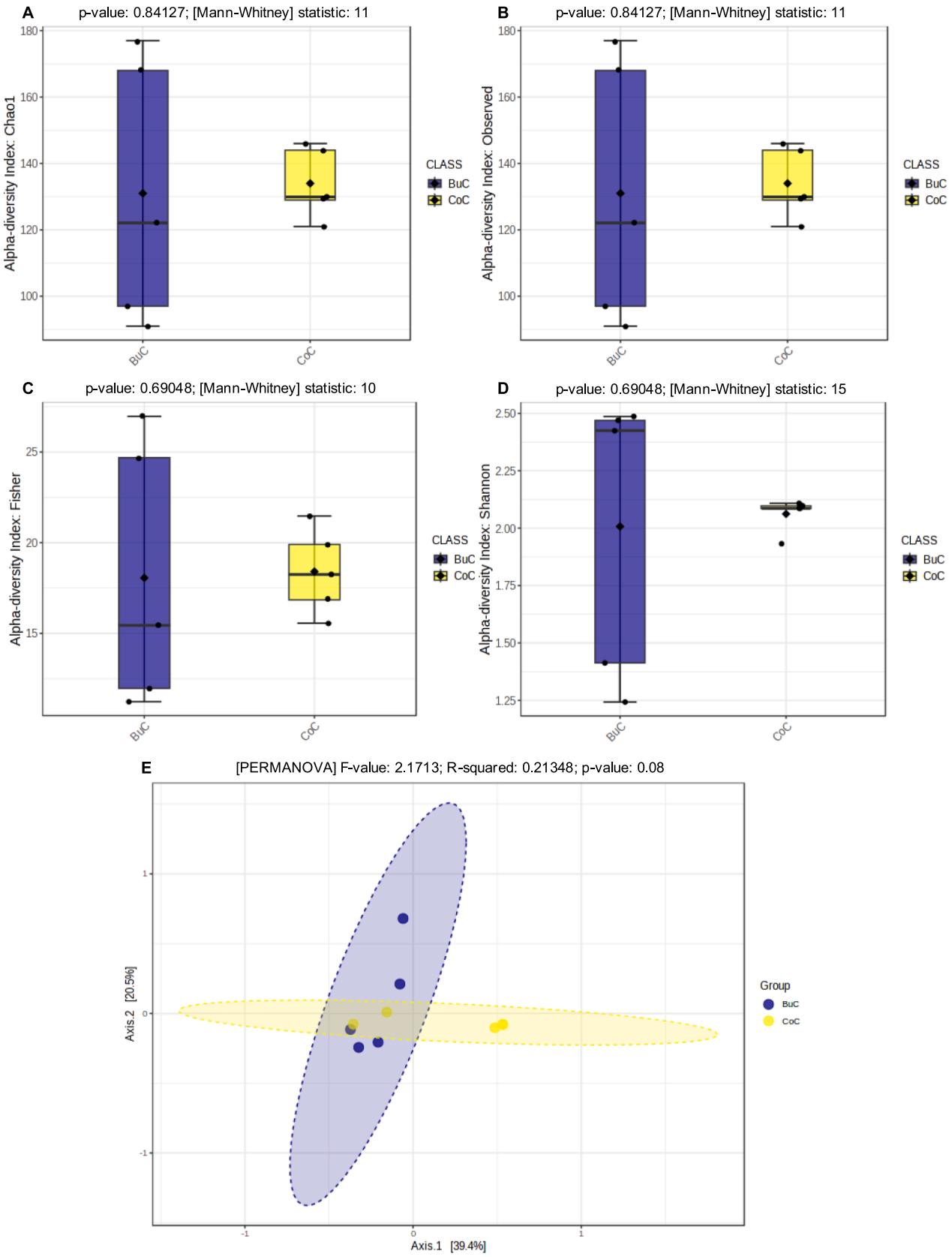

Fig. 1. Alpha and beta diversity analysis of bacterial communities in colostrum samples from buffalo and cow. Alpha diversity analysis: (A) chao1, (B) observed species, (C), Fisher index, and (D) Shannon diversity index. Beta diversity analysis: (E) principal coordinate analysis demonstrated variation in bacterial communities across samples. BuC, buffalo colostrum; CoC, cow colostrum.

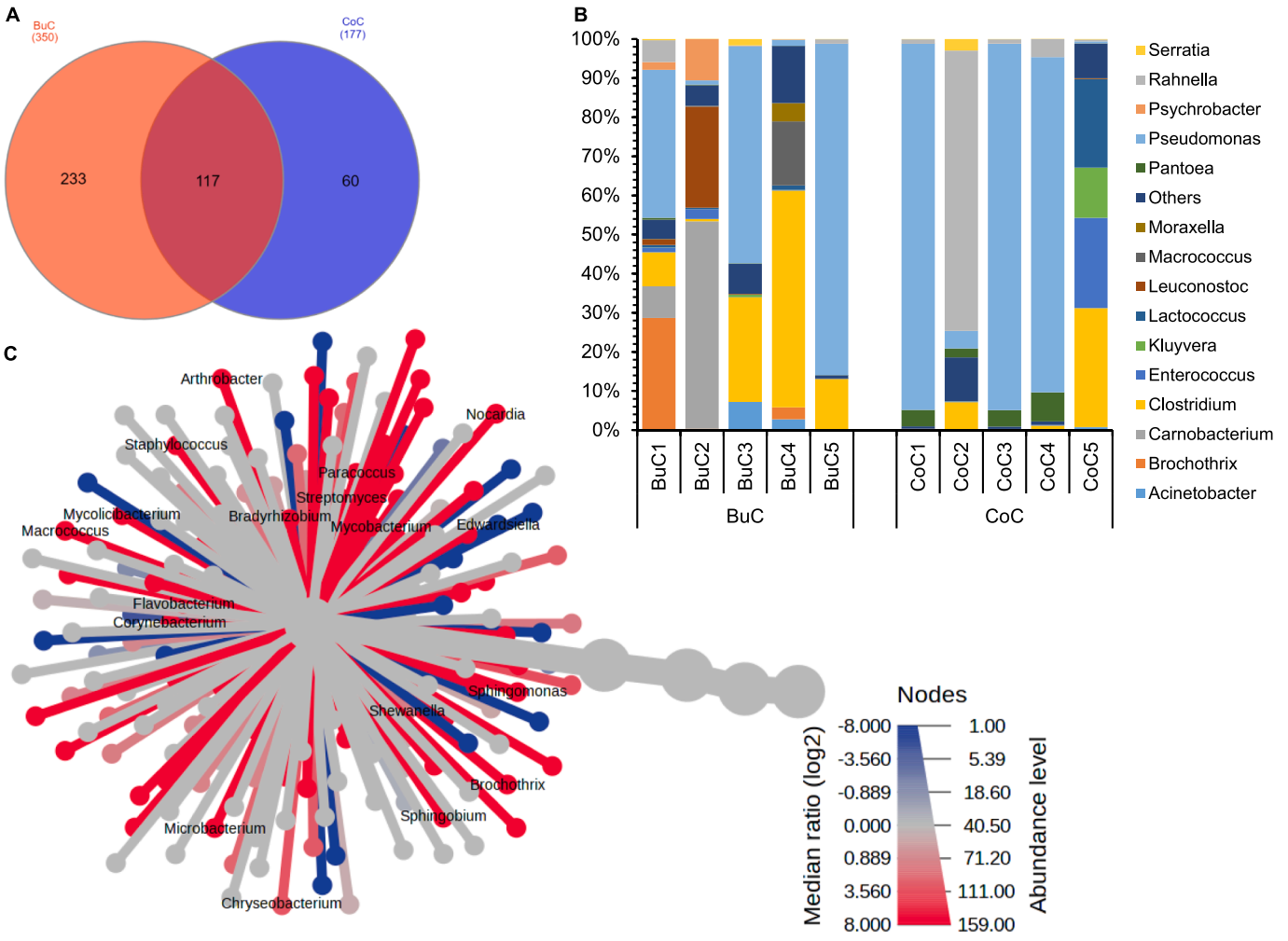

Fig. 2. Bacterial communities' analysis in colostrum samples from buffalo and cow at the genus taxonomic level. (A) Venn diagram analysis for the common and unique bacterial genera between BuC and CoC, (B) percentage relative abundance of the dominant genera in the colostrum samples, and (C) the heat tree illustrating the differences in genera between BuC and CoC using the Wilcoxon Rank Sum test. The color gradient and the size of the node, edge, and label are determined by the log2 ratio of median abundance. BuC, buffalo colostrum; CoC, cow colostrum.

aeruginosa, *Escherichia coli*, *Listeria monocytogenes*, and *Klebsiella pneumoniae* (Table S2). However, most of the clinically significant pathogenic bacteria were present at <1.0 % abundance in all colostrum samples, except for *C. botulinum*, which was commonly found at relatively high abundance in most of the samples. The opportunistic pathogenic bacterium *P. agglomerans* was identified at a relatively high abundance in cow colostrum samples (7.0 % ± 6.4 %) (Table S2).

3.4. MAGs taxonomic and phylogenetic analysis

The MAGs were recovered from the metagenomic assemblies and subsequently assessed for quality. Among the 29 MAGs, 11 exhibited genomes that were over 90 % complete with less than 15 % contamination, and 5 were classified as medium-sized MAGs, displaying ≥60 % genome completeness and less than 15 % contamination (Table S3). Phylogenetic analysis unveiled connections of the MAGs with eight species, while three MAGs were unclassified at the species level, including one from the genus *Psychrobacter* and two from the genus *Pantoea* (Fig. 4). FastANI was employed for taxonomy assignment and to evaluate novelty of the MAGs. It was found that three of the MAGs from cow colostrum samples were classified as *Pseudomonas carnis*, and two of the unclassified MAGs (CoC1_002 and CoC3_002) were linked to the *Pantoea* genus (Fig. 4). Notably, the MAG CoC5_002 was classified as a prominent probiotic, *L. lactis* (Table S3). The MAG CoC4_002 was classified as *P. agglomerans*. In buffalo colostrum, two of the MAGs were identified as *Carnobacterium maltaromaticum*, and MAG BuC2_002 was identified as *L. mesenteroides*, a species of lactic acid bacteria associated with fermentation. The remaining MAGs from buffalo colostrum were phylogenetically linked with various species, including *M. caseolyticus*, *Rothia* sp. 002418375, *Brochothrix thermosphacta*, *Pseudomonas psychrophila*, and the unclassified MAG BuC2_004, which belonged to the genus *Psychrobacter* (Fig. 4).

3.5. MAGs functional analysis

From genome annotation, an average of 2654.8 ± 1230.8 protein-encoding genes with functional assignments and 1941.4 ± 1009.2 protein-encoding genes without functional assignments were identified in the contig assemblies of the identified MAGs (Table S3). The percentage of protein-encoding feature coverage for the identified MAGs ranged from 94.9 to 153.1. The functional analysis of the MAGs using the sub-system revealed that gene abundance was associated with various metabolic pathways, including amino acids metabolism, carbohydrate metabolism, cofactors, vitamins, prosthetic groups, energy and precursor metabolites generation, fatty acids, lipids, and isoprenoids biosynthesis, as well as membrane transport. Within amino acid metabolism, genes from pathways related to lysine, threonine, methionine, and cysteine were predominantly found, followed by genes

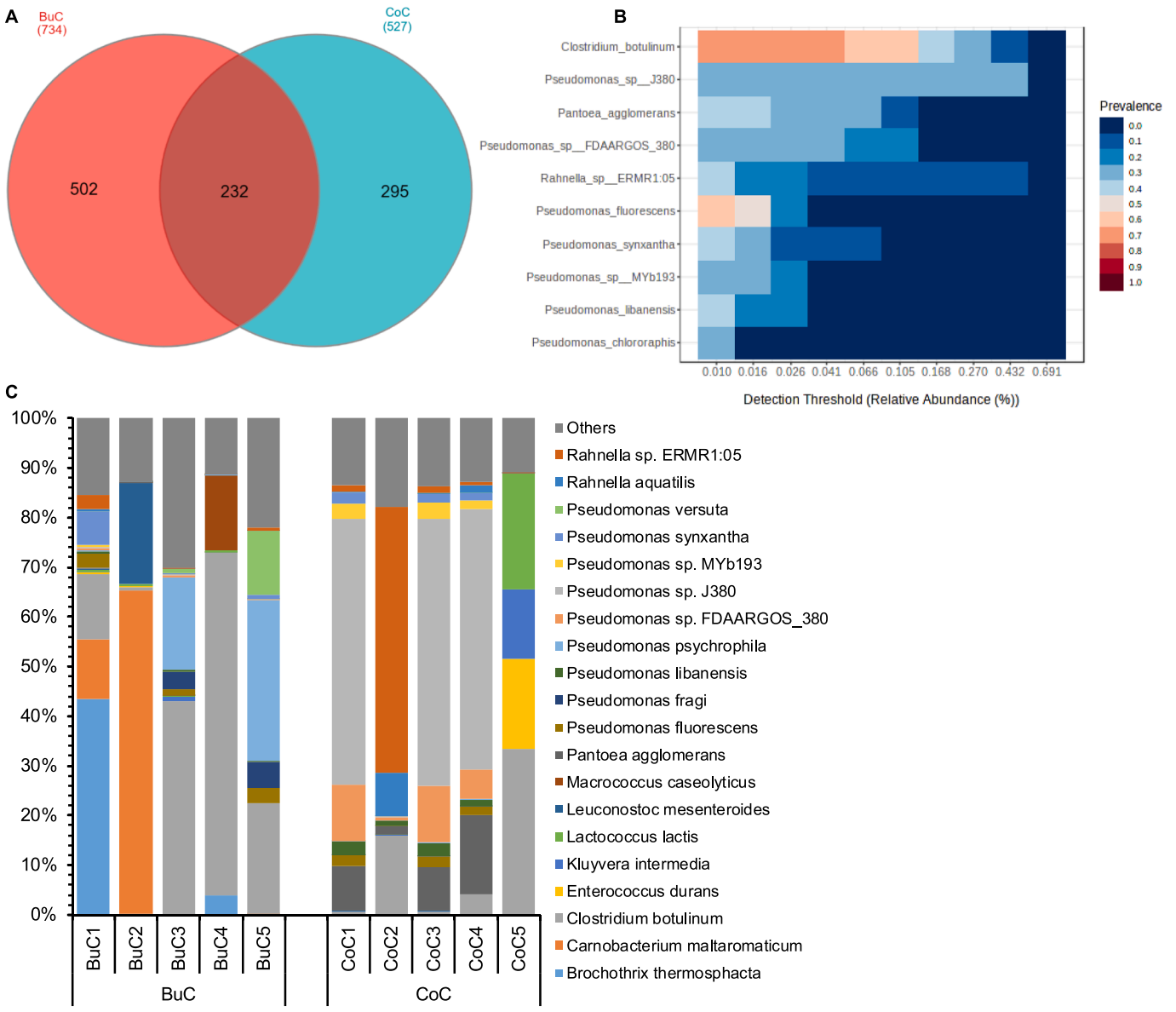

Fig. 3. Bacterial species analysis in colostrum samples from buffalo and cow. (A) Venn diagram analysis for the common and unique bacterial species between BuC and CoC, (B) core bacterial species, and (C) percentage relative abundance of the dominant species in the colostrum samples. BuC, buffalo colostrum; CoC, cow colostrum.

associated with arginine, the urea cycle, creatine, polyamines, and aromatic amino acids and derivatives (Table S3). The genes related to carbohydrate metabolism were classified into nine sublevels. The highest abundance of genes was linked to sugar alcohols, monosaccharides, and C-1 compound metabolism (Table S3). The fermentation genes were categorized into three groups: mixed acid fermentation, lactate fermentation, and acetoin, butanediol metabolism. The cofactors and vitamin synthesis genes were further categorized into 11 sublevels, including folate and pterines, biotin, lipoic acid, pyridoxine, and riboflavin (Table S3). Additionally, genes related to fatty acids and isoprenoids were found at relatively high abundance in the MAGs genomes (Table S3).

3.6. Antimicrobial resistance genes analysis

Using the ARGs-OAP tool analysis with criteria of 80 % identity, 175 ARGs and variants were detected, with 55 ARGs common to both buffalo and cow colostrum metagenomes. Notably, an increased diversity in the distribution of ARGs was found among buffalo samples, which carried six common ARGs (Fig. S4A). In contrast, 24 ARGs were common among all the studied cow samples (Fig. S4B). In the core resistome analysis, 11 ARGs (*bacA*, *arnA*, *mexF*, *mexB*, *msbA*, *mexK*, *Acinetobacter baumannii abaQ*, *mdtK*, *opmH*, *oprN*, and *mexE*) were found to be present in at least 50 samples of each colostrum type (Fig. 5). In the PCoA analysis, the ARGs from the two colostrum types were clustered separately, but the difference was not found to be statistically significant ($p = 0.2$) (Fig. S4C). The most common mechanism of antimicrobial resistance in both types of colostrum metagenomes was antibiotic efflux, which included 87 genes and variants, primarily associated with multidrug resistance. Among other resistance mechanisms, 56 ARGs were linked to antibiotic inactivation, mainly conferring resistance to beta-lactam and aminoglycoside antibiotics. Additionally, 16 ARGs were associated with the antibiotic target protection mechanism, and 10 ARGs were linked to antibiotic target alteration, primarily causing resistance to peptide antibiotics. The ARGs associated with antibiotic efflux and antibiotic target alteration mechanisms were relatively more abundant in cow colostrum metagenomes compared to buffalo.

The bacitracin-resistance *bacA* gene was identified at relatively high

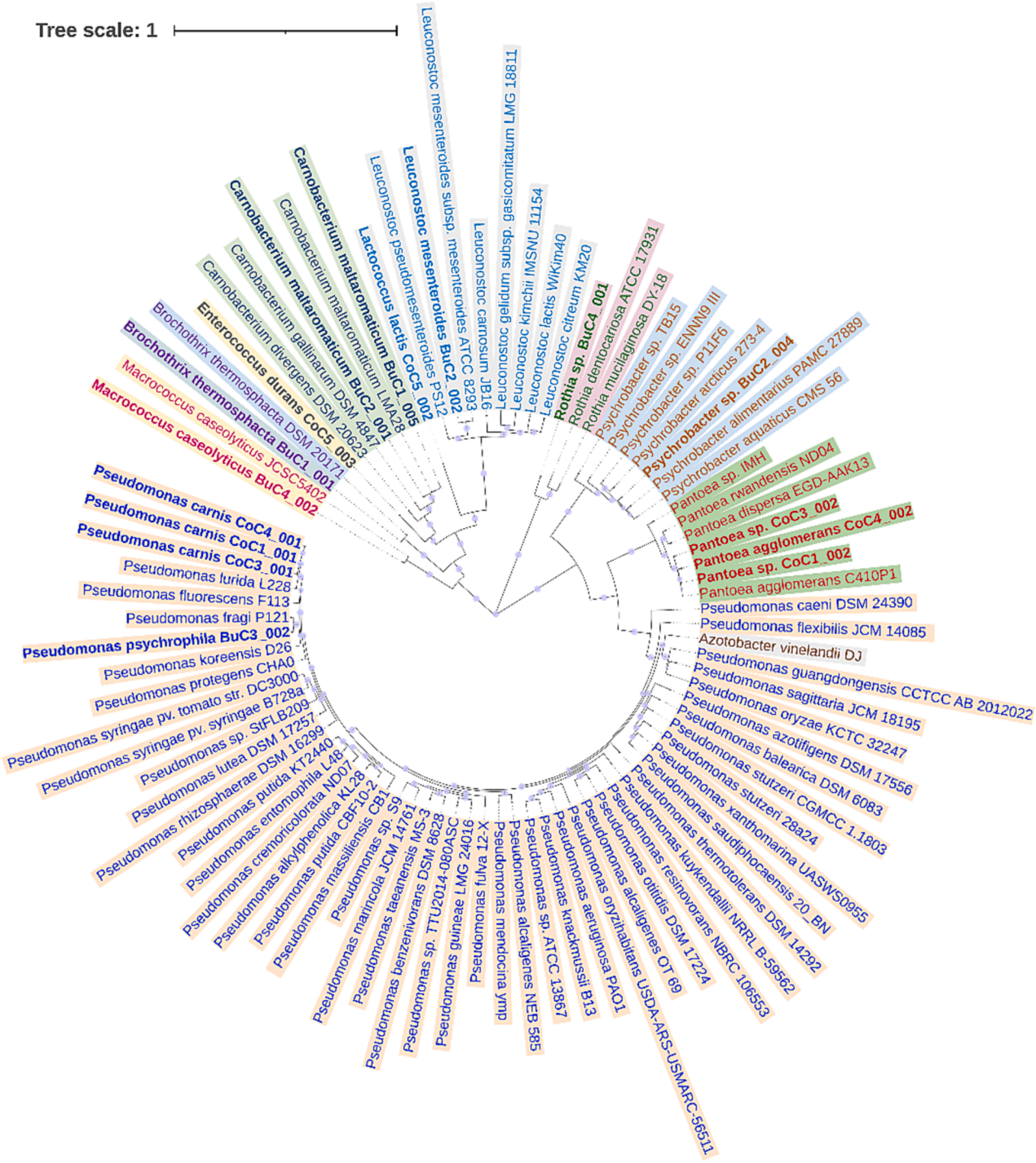

Fig. 4. Maximum likelihood phylogenetic analysis of metagenome-assembled genomes (MAGs) retrieved from buffalo and cow colostrum. Closely related genomes of bacterial species retrieved from GenBank. Genomes from this study are denoted in bold font.

abundance in both colostrum types (Fig. 5). There was noticeable inter and intra-sample variation in the relative abundance of ARGs in the samples from both colostrum types. For instance, aminoglycoside resistance in buffalo samples was found to be 66.4 ± 54.2 rpkm, while in cow samples, it was 4.5 ± 9.8 rpkm. Quinolone resistance in buffalo samples was 25.2 ± 24.7 rpkm, whereas in cow samples, it was 11.7 ± 11.9 rpkm. Polymyxin resistance in buffalo samples was 21.2 ± 17.0 rpkm, while in cow samples, it was 97.2 ± 80.8 rpkm. Beta-lactam resistance in buffalo samples was 3.4 ± 3.4 rpkm, and in cow samples, it was 18.6 ± 23.8 rpkm. Resistance genes for tetracycline and phenicol antibiotics were only found in buffalo samples. Pairwise comparison using the Wilcoxon rank sum test revealed that the AMR class aminoglycoside was significantly different ($p = 0.03$) between the buffalo and cow colostrum, and ARGs for antibiotic target replacement were significantly more abundant ($p = 0.01$) in buffalo samples compared to cow samples. The Pearson r correlation indicated that resistance to AMR classes aminoglycoside, tetracycline, and sulfonamide were positively correlated with buffalo colostrum compared to cow. On the other hand, multidrug resistance, peptide resistance, macrolide resistance, and beta-lactam resistance were positively correlated with cow colostrum compared to buffalo.

Utilizing perfect and strict matches with the CARD, a total of 23

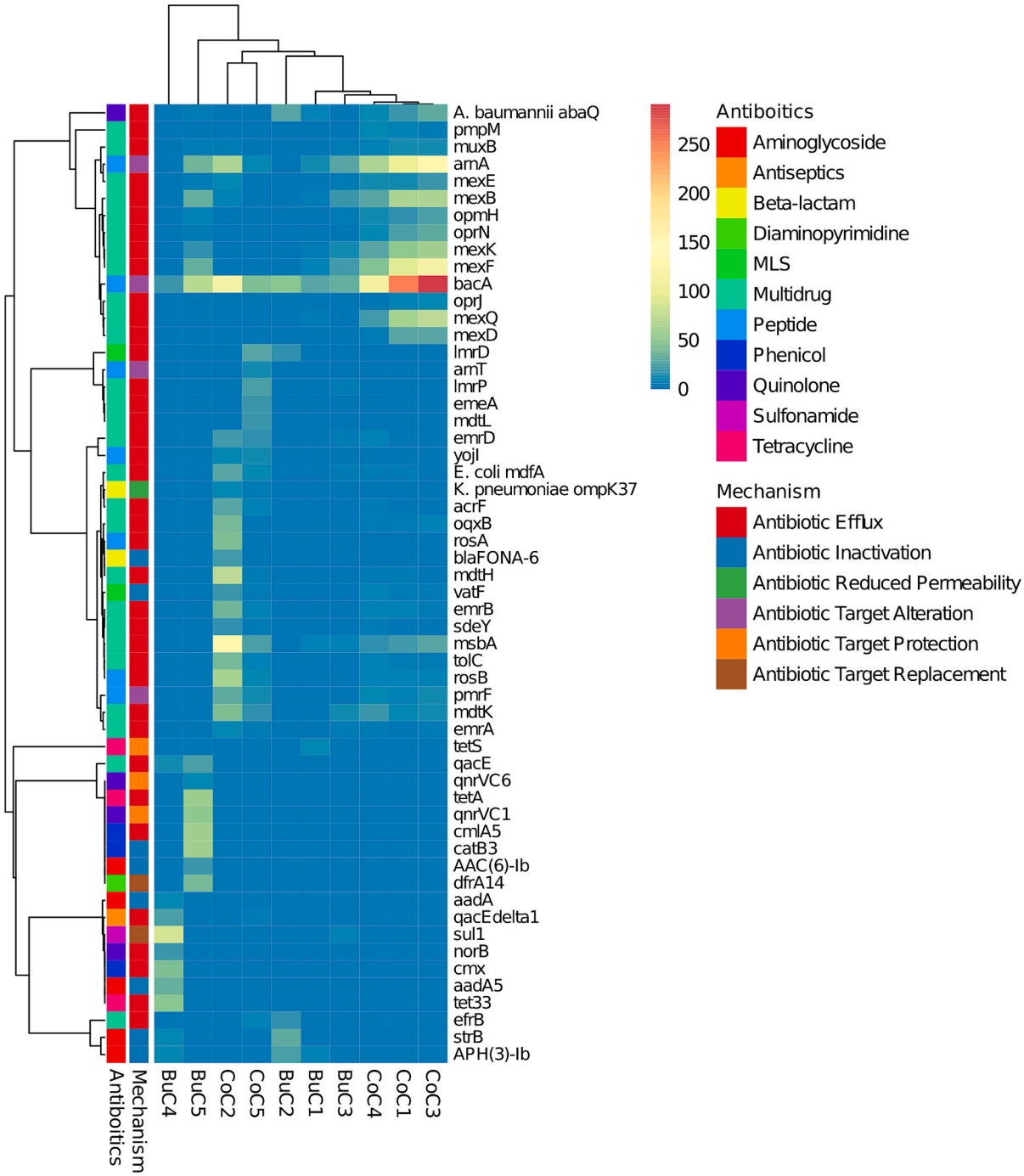

Fig. 5. Antimicrobial resistance genes (ARGs) identified from shotgun sequencing in abundance of ≥10 rpkm at least in one colostrum sample. ARGs counts normalized to reads per kilobase per million (rpkm). BuC, buffalo colostrum; CoC, cow colostrum; MLS, macrolides, lincosamides, and streptogramins.

ARGs and their variants were identified in the MAGs. These matches exhibited a sequence identity of ≥85 %, as determined through the blast analysis of MAGs (Fig. S5). An increased number of eight ARGs were detected in each unclassified species of *Pantoea*, and six ARGs were detected in each *P. carnis* MAG retrieved from cow colostrum (Fig. S5). There was a relatively decreased number of ARGs detected in the MAGs retrieved from buffalo compared to cow. In line with ARGs-OAP results, the ARGs detected from MAGs were associated with resistance against clinically important antibiotics such as aminoglycoside, fluoroquinolone, tetracycline, sulfonamide, and peptide antibiotics.

3.7. Colostrum mycobiome and virome analysis

From the blast analysis against the fungal database of the sequence reads obtained from shotgun sequencing of colostrum samples, an average of 3.1 % ± 3.9 % were classified as fungi. A relatively high abundance of fungal reads was observed in two of the buffalo colostrum samples: BuC1 (10.8 %) and BuC2 (10.2 %). Fungal-associated reads were further classified at the species taxonomic level, and the percentage relative abundance was calculated. In total, 322 fungal species were found in colostrum samples. There was a relatively larger number of species identified in buffalo colostrum (288) samples compared to cow colostrum (265) samples (Fig. 6A). Among the identified species, 231

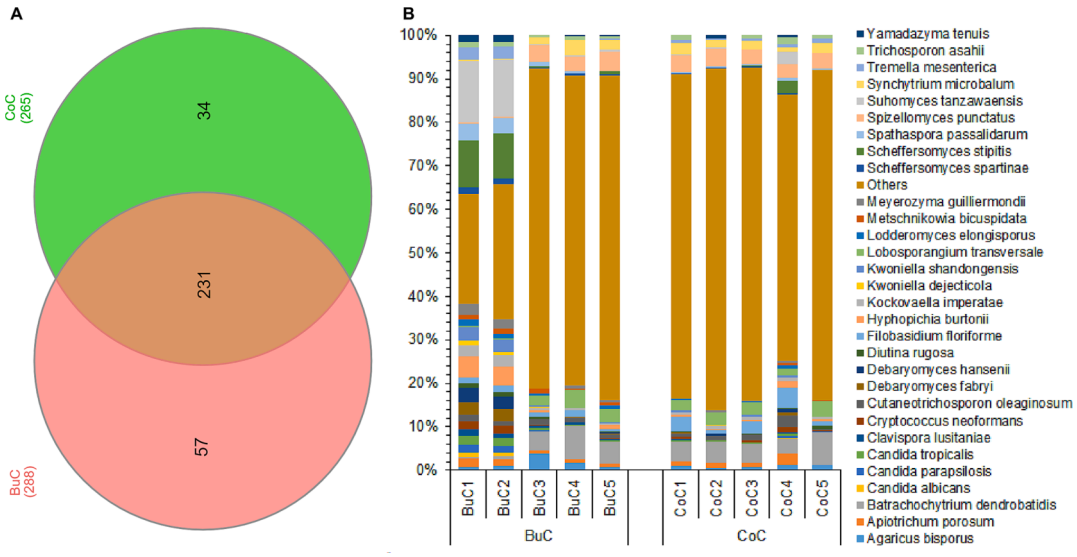

Fig. 6. Distribution of fungal species in the colostrum samples from buffalo and cow. (A) Venn diagram analysis depicting the common and unique fungal species between BuC and CoC, and (B) the percentage abundance of the relatively dominant fungal species in the colostrum samples. The relative abundance was computed by normalizing the sequence reads of each fungal taxon. This normalization was achieved by dividing the sequence reads of each fungal taxon by the total number of fungus-associated sequence reads in the corresponding metagenome. BuC, buffalo colostrum; CoC, cow colostrum.

species were common to both animal colostrum, while 57 species were unique to buffalo, and 34 were exclusively detected in cow colostrum (Fig. 6A). Furthermore, 29 species were identified that were present in at least 80 % of the samples, including *Batrachochytrium dendrobatidis, Spizellomyces punctatus, Lobosporangium transversal, Rhizopus microspores*, and *Synchytrium microbalum* (Fig. 6B). Among the dominant species detected in colostrum samples were *Filobasidium floriforme, Scheffersomyces stipites, Suhomyces tanzawaensis, Hyphopichia burtoniim, Spathaspora passalidarum, Linderina pennispora, Sordaria macrospora,* and *Meyerozyma guilliermondii*. The relative abundance distribution of the common and dominant fungal species varied among buffalo and cow colostrum samples (Fig. 6B).

The virome from colostrum samples was studied based on the metagenomes using the Reference Viral Database (RVDB). Only 1.1 % ± 0.9 % sequence reads in the colostrum samples were related to viruses, and they were classified into 55 viruses (Table S4). Only eight viruses were found that were present in ≥60 % of the tested samples including Orf virus, Mimiviridae sp. ChoanoV1, Chlorovirus, Syngen Nebraska virus 5, Pandoravirus, Catovirus CTV1, and uncultured virus. Among them Orf virus, a member of the parapoxvirus was commonly found in all the samples at relatively high abundance followed by the uncultured virus (Table S4).

4. Discussion

Animal colostrum consumption by humans is gaining popularity due to its beneficial contents such as vitamins, growth and immune factors, nutrients, and antibodies. Potential probiotic properties further favor the consumption of animal colostrum. However, colostrum's microbiological and nutritional composition can rapidly change in response to the type of parturition, stress, and environmental factors (Drago et al., 2017). Bacterial contamination and the transmission of antimicrobial resistance are risk factors associated with the consumption of raw colostrum (Miranda et al., 2023; Morrill et al., 2012; Slosarkova et al., 2021). It is important to screen the animal colostrum samples for microbiological safety along with probiotic potential. This study examined the microbiome composition of rarely studied buffalo colostrum and compared it with frequently investigated cow colostrum. The colostrum microbiomes were comprehensively analyzed by employing the shotgun sequencing technique. However, a small sample size remained the limitation of this study.

Buffalo and cow colostrum exhibit a diverse composition of bacteria, primarily classified into the phyla Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria, as documented in prior studies (Hang et al., 2020; Vasquez et al., 2022). However, the relative abundance of these phyla substantially varied in the colostrum of both animals and within colostrum samples of each animal. Cumulatively, Firmicutes and Proteobacteria were more abundant in buffalo and cow colostrum samples, respectively. Firmicutes were abundant in buffalo and Proteobacteria were dominated in cow colostrum. Interestingly, the same phyla are also prevalently found in sow and human milk (Chen et al., 2018; Kumar et al., 2016; Moossavi et al., 2019). The source of these phyla has been extensively studied but remains not fully understood. Generally, environmental bacteria, maternal skin bacteria, and neonate's oral cavity bacteria are considered the main sources of bacterial presence in the mammary gland (Derakhshani et al., 2018; Slosarkova et al., 2021). Nevertheless, the presences of anaerobic intestinal microbiota-related bacterial species in the mammary gland are linked to their translocation from the gut during late gestation and lactation stages. This phenomenon is mentioned as an entero-mammary pathway in humans (Selvamani et al., 2021) and has also been proposed in cows and other ruminants (Greiner et al., 2022; Young et al., 2015).

The core genera *Pseudomonas, Rahnella, Clostridium, Enterococcus*, and *Pantoea* were commonly identified in cow and buffalo colostrum. However, the relative abundance of other genera such as *Macrococcus*, *Paracoccus*, *Nocardia*, *Brochothrix*, *Arthrobacter*, *Streptomyces*, *Corynebacterium*, *Sphingobium*, *Microbacterium*, and *Edwardsiella* significantly varied in the colostrum of both animals. Similarly, previous investigations on cow colostrum reported varying relative abundance of these genera through 16S amplicon sequencing analysis (Chen et al., 2021; Drago et al., 2017). Bonsaglia et al. identified 30 bacterial genera in cow milk samples, including *Psychrobacter*, *Corynebacterium*, *Staphylococcus*, *Acinetobacter*, and *Arthrobacter* (Bonsaglia et al., 2017). Chen et al. did not observe statistically significant differences in the relative abundance of most core genera (Chen et al., 2021). However, the core taxa identified in colostrum samples are varied in different studies such as *Bacillus, Bacteroides, Staphylococcus, Acinetobacter*, and *Pseudomonas* reported in few studies but different from the finding of this study and other studies (Chen et al., 2021; Slosarkova et al., 2021). *Pseudomonas* are psychrotrophic microorganisms and are common contaminants in

the dairy environment, as suggested in previous studies (Drago et al., 2017; Slosarkova et al., 2021). However, the origin of *Pseudomonas* in raw milk and colostrum samples is not exactly known. Therefore, the implementation of hygienic procedures during the milking process is crucial to reduce contamination with *Pseudomonas* and other environmental microorganisms.

The results revealed the detection of *Pseudomonas* species, *Rahnella* sp. ERMR1:05, *C. botulinum*, and *P. agglomerans* core species in colostrum samples with substantially differentiating relative abundance in buffalo and cow colostrum. Specifically, the abundance of *Pseudomonas* species remained high in cow colostrum samples. MAGs of *P. agglomerans* and *P. carnis* were retrieved from cow colostrum's metagenomic assemblies whereas MAGs of *C. maltaromaticum*, *B. thermosphacta*, and *L. mesenteroides* were detected in buffalo colostrum samples. The Chao1 and Shannon index demonstrated an increased intra-sample variation in buffalo colostrum. Previous studies have consistently reported varying colostrum microbiomes, which could be due to different diets, environments, and management practices (Drago et al., 2017; Hang et al., 2020; Lima et al., 2017). A study in China investigated bacterial community profiles of two dairy farms in different areas and reported significantly different colostrum bacterial structures in both farms (Chen et al., 2021).

During this study, probiotics such as *S. thermophilus*, *L. mesenteroides*, *L. garvieae*, *E. faecium*, *L. lactis*, *L. fermentum*, and *L. plantarum* were identified in both colostrum from cow and buffalo. However, none of these probiotics belonged to core bacteria. Multiple studies reported probiotics (e.g., *L. lactis*, *Bifidobacterium crudilactis*, and *L. paracasei*) distribution in human colostrum and cow milk (Bagci et al., 2019; Damaceno et al., 2017; Quigley et al., 2013). Nevertheless, these probiotic taxa were not commonly regarded as core colostrum microbiome taxa (Chen et al., 2021). Lyons et al. stated that exploration of unconventional bacterial species could facilitate probiotics development (Lyons et al., 2020). For example, bacteria belonging to Sphingobacteriaceae, found in colostrum of both cow and buffalo, previously reported to produce sphingolipids, which known for their positive effects on infants, enhancing both gut health and immunity (Nilsson, 2016).

We observed numerous pathogenic and opportunistic pathogenic bacteria in buffalo and cow colostrum. Most buffalo colostrum samples predominantly contained *C. botulinum* whereas other pathogenic bacteria such as *K. pneumoniae*, *P. aeruginosa*, *Acinetobacter baumannii*, *E. coli*, and *S. aureus* presented a minor relative abundance. Similarly, pathogenic, and opportunistic pathogenic bacteria like *Streptococcus pneumoniae, P. aeruginasa, Delftia tsuruhatensis, Stenotrophomonas maltophilia, Staphylococcus*, and *E. coli* were detected in previous studies from healthy cows' colostrum (Chen et al., 2021; Lima et al., 2017). The distribution of pathogenic bacteria in regular milk or colostrum is considered harmful to newborns. Therefore, several Chinese dairy farms prefer feeding pasteurized milk to calves to mitigate the risk of infections associated with intestinal pathogens. The pasteurization process helps to alleviate intestinal infections; however, it may interfere with the development of the calf's gut microbiota. Recent studies established that pathogen-exposed animals exhibit higher resistance to succeeding infections (Stacy et al., 2021). In addition to vaccination, newborns also develop innate and adaptive immunity through other microbial exposures, such as those occurring in the uterus and through diet (Macpherson et al., 2017). Further studies involving large sample sizes could confirm the *C. botulinum* dominance in colostrum samples.

Different farming practices, inter-individual microbiota variations, and contamination risks complicate the milk and animal colostrum microbial flora investigations (Addis et al., 2016; Carafa et al., 2020; Kumar et al., 2016). Milk microbiota contains microbes originating from extramammary sites and mammary glands. Maternal intestinal microbiota has also been suggested as the major microorganism source of breast milk (Macpherson and Uhr, 2004; Young et al., 2015). Macrophages and dendritic cells can capture gut bacteria, which are translocated to the mesenteric lymph nodes, and distant organs including the mammary gland (Macpherson and Uhr, 2004). Chen et al. observed over 50 % of the Operational Taxonomic Units identified in colostrum were also found in fecal samples (Chen et al., 2021). Notably, certain strict gastrointestinal tract anaerobes such as *Clostridium butyricum* and *Bacteroides fragilis* have been previously detected in colostrum, which supports the hypothesis that intestinal bacteria could be the source of strict anaerobes found in colostrum (Lima et al., 2017). Environment, fecal contamination, and external sites of the body are also known sources of milk anaerobes (Henderson et al., 2015; Taponen et al., 2019). *C. botulinum* is frequently found in the environment of animal farms and in the animal gut reported in previous studies (Bohnel and Gessler, 2013; Lindstrom et al., 2010). It could be a probable cause of the high abundance observed in colostrum samples in this study. Several environmental bacterial genera including *Psychrobacter*, *Arthrobacter*, and *Acinetobacter* found in soil, water, and various habitats, have been reported in milk (Taponen et al., 2019). In lactating animals, the udder is open, allowing bacteria from the outside to enter the mammary gland through the teat canal and transfer to the milk.

In this study, the microbiome analysis of the colostrum samples (buffalo and cow) taxonomically classified most of the sequence reads as bacteria followed by fungi (3.1 % ± 3.9 %), and DNA viruses (1.1 % ± 0.9 %). Previously, a limited number of studies performed mycobiome and virome analysis of animal colostrum samples, particularly from buffalos and cows. Consistent with bacterial community analysis, no significant difference was observed in the alpha and beta diversity of mycobiome in buffalo and cow colostrum. Contrarily to bacteria, fungal communities displayed more coherence in the colostrum of both animals. The colostrum of both animals shared 231 fungal species out of the total 322 species. Similar to human breast milk, *Rhodotorula*, *Malassezia*, *Candida*, *Aspergillus*, and *Penicillium* genera were found in buffalo and cow colostrum at varying relative abundance (Boix-Amoros et al., 2019).

Food-based entry of ARGs into the body could reach the gut microbiome and exert adverse health impacts. This study detected 175 ARGs and related variants, which are not only producing resistance to most clinically important antibiotic classes but also causing multidrug resistance. In other studies, ARGs have also been observed in human, and animal milk and colostrum microbiomes that mitigate the efficiency of antibiotic classes including fosfomycin, aminoglycosides, beta-lactam, nitroimidazoles, fluoroquinolones, rifamycins, tetracycline, monobactam, lincosamides, phenicols, macrolides, and nitrofurans. The detection of a higher relative abundance of bacitracin resistance-related *bac*A gene could be associated with bacitracin producing concentration from colostrum specific taxa as reported in a previous study (Bagci et al., 2019). The phenotypic activities of the AGRs, identified from shotgun sequencing data, could not be confirmed through a metagenomic approach and required further investigation. The highest number of ARGs linked to MDR, penam, cephamycin, peptide antibiotics, phosphonic acid, elfamycin, and cephalosporin resistance were detected in the species belonging to genus *Pantoea* whereas peptide antibiotics and fluoroquinolone-associated six ARGs were identified in *P. carnis* MAGs retrieved from cow colostrum. Consistent with the metagenomic data, comparatively lower numbers of ARGs were noted on the MAGs retrieved from the buffalo colostrum. The detection of ARGs related to fluoroquinolones, beta-lactam, aminoglycoside, and cephalosporin resistance might be due to the usage of these antibiotics in the farms. However, the increased number of MDR-associated ARGs, causing resistance through the efflux mechanism, could be the result of intersection to the colostrum microbiome. In a previous study, $bla_{\text{TEM-171}}$ and $bla_{\text{CTX-M-15}}$ genes reported in ESBL-producing *E. coli* recovered from colostrum fed to dairy calves, and the isolate demonstrated high resistance to kanamycin, tetracycline, ampicillin, and ciprofloxacin (He et al., 2021).

5. Conclusions

The shotgun metagenomic sequencing provided comprehensive insights into the microbiota of buffalo and cow colostrum collected from rural sites in Pakistan. The results highlighted the variations in microbial diversity and associated ARGs of buffalo and cow colostrum. Taxonomically, the core microbiome remained consistent in cow and buffalo colostrum but presented significant inter and intra-sample variations in the dominant colostrum microbial flora of both animals. The differences in the colostrum microbiome could be ascribed to the environmental factors and physiological and genetic differences between buffalos and cows. In addition to probiotic species, a large number of pathogenic and opportunistic pathogenic bacteria were also noticed in buffalo colostrum. The dominant abundance of *C. botulinum*, noted during this study, requires further confirmation through a large sample size. The study recommends that nutritional value and microbiological safety, particularly of raw colostrum, should be evaluated in developing countries before consumption.

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CRediT authorship contribution statement

Muhammad Yasir: Conceptualization, Methodology, Formal analysis, Funding acquisition, Writing – review & editing. **Ibrahim A. Al-Zahrani:** Resources, Methodology, Data curation, Writing – review & editing. **Raees Khan:** Data curation, Formal analysis. **Samah Abdullah Soliman:** Conceptualization, Resources, Writing – review & editing, Funding acquisition. **Safaa A. Turkistani:** Conceptualization, Funding acquisition, Writing – review & editing. **Maha Alawi:** Resources, Methodology, Formal analysis, Writing – review & editing. **Esam I. Azhar:** Conceptualization, Visualization, Resources, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The shotgun sequencing data submitted to NCBI Sequence Read Archive under BioProject ID: PRJNA1048012.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2024.103957.

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