Einfluss peri-operativer Antibiotika Regime auf Häufigkeit und lokale Dynamik ESBL-positiver *Escherichia coli* sowie Komposition und Diversität des enteralen Mikrobioms bei hospitalisierten Pferden

I n a u g u r a l d i s s e r t a t i o n

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Vor den Erfolg haben die Götter den Schweiß gesetzt

(Weisheit meiner Oma)

nach Hesoid 715 - 650 v. Chr

Inhaltsverzeichnis

Abkürzungsverzeichnis

5DG	(engl.) 5 day group
AcrB	Acriflavin-Resistenz-Protein B, Effluxproteinkomplex mit breiter Substratspezifität
AmpC-β-Laktamase	Enzym mit *AmpC*-Gen vermittelter Resistenz gegen Penicilline, Cephalosporine der zweiten und dritten Generation, Cephamycine, sowie β-Laktamase-Inhibitoren
AMR	Antimikrobielle Resistenz
BEL	β-Laktamase, Ableitung: (engl.) Belgium extended β-lactamase
Bspw.	Beispielsweise
CLSI	Clinical and Laboratory Standards Institute
CTX-M	β-Laktamase, Ableitung: Aktivität gegen Cefotaxim, erstmals isoliert in München
DART	Deutsche Antibiotika-Resistenzstrategie
DNA	(engl.) Desoxyribonucleic acid
E. coli (EC)	(lat.) *Escherichia coli*
etc.	(lat.) *et cetera*, und so weiter
engl.	Englisch
ESBL	(engl.) Extended-Spectrum-β-Lactamase
ESBL-EC	ESBL produzierende *Escherichia coli*
et al.	(lat.) *et alii*, und andere
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GES	β-Laktamase, Ableitung: (engl.) Guiana-extended spectrum
GIT	Gastrointestinaltrakt
HAI	Hospital-assoziierte Infektionen
HGT	Horizontaler Gentransfer
K. oxytoca	(lat.) *Klebsiella oxytoca*
K. pneumoniae	(lat.) *Klebsiella pneumoniae*
Konz.	Konzentration
lat.	Lateinisch
MHK	Minimale Hemmkonzentration
MRE	Multiresistenter Erreger
MRSA	Methicillin-resistenter *Staphylococcus aureus*
NCBI	National Center for Biotechnology Information
NGS	(engl.) Next Generation Sequencing
nm	Nano Meter (Einheit)
OD_{600}	Optische Dichte (für Zelldichte Bestimmungen bei 600nm Extinktion)
OHHLEP	One Health High Level Expert Panel
OTU	(engl.) Operative Taxonomic Unit
OXY	β-Laktamase, Ableitung: gefunden in *K. oxytoca*
P/G	Penicillin/Gentamicin
PAP	Perioperative antibiotische Prophylaxe
PBP	Penicillin-Bindeprotein
PCA	(engl.) Principal Component Analysis
PCR	(engl.) Polymerase Chain Reaction
PDR	Panresistent

PER	β-Laktamase, Ableitung: (engl.) Pseudomonas extended resistant; sowie Initialen der Entdecker: Patrice, Esthel und Roger
RNA	(engl.) Ribonucleic acid
RND	(engl.) Resistance Nodulation-Devision
S. aureus	(lat.) *Staphylococus aureus*
SCCmec	(engl.) Staphylococcal Cassette Chromosome mec
SDI	(engl.) Shannon Diversity Index
SHV	β-Laktamase, Ableitung: (engl.) Sulfhydryl Reagent Variable
spp.	(lat.) *Species Pluralis,* mehrere Arten
SSG	(engl.) Single Shot Group
SSI	(engl.) Surgical Site Infections
ST	Sequenztyp
STC	Sequenztypkomplex
TEM	β-Laktamase, Ableitung: Benannt nach dem Patienten (Temoneira), welcher die erste Probe lieferte
TLA	β-Laktamase, Ableitung: Benannt nach den Tlahuicas-Indianern
u.a.	Unter anderem
VEB	β-Laktamase, Ableitung: (engl.) Vietnam Extended-Spectrum β-Laktamase
WHO	(engl.) World Health Organization, Weltgesundheitsorganisation
XDR	Weitgehend resistent
z.B.	Zum Beispiel
z.T.	Zum Teil

Zusammenfassung

Diese Arbeit war Teil eines vom Bundesministerium für Bildung und Forschung geförderten interdisziplinären Forschungsnetzwerks (#1HealthPREVENT) und stellte eine einmalige peri-operative antibiotische (Penicillin/Gentamicin (P/G)) Prophylaxe (PAP) im Zuge eines operativen Eingriffs nach diagnostizierter Kolik beim Pferd der bislang üblichen fünf-Tage-Antibiose gegenüber, mit dem Ziel den Einfluss der PAP auf die Häufigkeit von (engl.) Extended Spectrum β-Lactamase produzierenden *Escherichia coli* (ESBL-EC) und die Veränderungen im enteralen Mikrobiom der Pferde zu untersuchen und zur Verbesserung des sorgfältigen Einsatzes von Antibiotika in der Veterinärmedizin beizutragen. Die per Los jeweils einer der zwei Gruppen („single shot“ Gruppe (SSG); „5 days“ Gruppe (5DG)) zugeordneten Pferde wurden dafür jeweils an drei verschiedenen Zeitpunkten (Klinikaufnahme (t_0), Tag 3 (t_1) und Tag 10 (t_2) postoperativ) beprobt (Kotproben und Nüsternabstriche). Zusätzlich zur Gruppe der hospitalisierten Pferde wurde auch eine nicht-hospitalisierte Kontrollgruppe ohne klinische Auffälligkeiten einbezogen. Alle Proben wurden hinsichtlich positiver ESBL-EC untersucht und die identifizierten Isolate phänotypisch (durchgeführt vom Institut für Mikrobiologie und Tierseuchen, Freie Universität Berlin) und genotypisch charakterisiert. Unabhängig vom P/G PAP-Schema stieg für die Pferde die Wahrscheinlichkeit von t_0 zu t_1 sowie von t_0 zu t_2 an, positiv für ESBL-EC zu sein. Die Ganzgenom-Sequenzierung der Isolate ergab außerdem eine enge räumliche und zeitliche Beziehung zwischen Isolaten mit gemeinsamen Sequenztypen, was auf eine lokale Ausbreitung hindeutete. Die 16S rRNA-Gen Sequenzierung der Kotproben (durchgeführt vom Institut für Klinische Molekularbiologie der Christian-Albrechts-Universität zu Kiel) zur Untersuchung der Veränderungen im enteralen Mikrobiom zeigte nach der bioinformatischen Aufbereitung (durchgeführt von Silver Anthony Wolf, Robert Koch-Institut) und Fach-übergreifenden Analyse eine Beeinträchtigung in der Zusammensetzung der fäkalen Mikrobiota (Alpha-Diversität) für Pferde mit akuter Kolik im Vergleich zur Kontrollgruppe, welche jedoch nicht signifikant war. Die mikrobielle Gesamtkomposition der untersuchten Proben (Beta-Diversität) wies vor allem für die 5DG an t_1 erhebliche Einschränkungen auf, was höchstwahrscheinlich auf die fortlaufende Verabreichung von Antibiotika zurückzuführen war. In beiden Studiengruppen wurde zudem an t_1 eine erhöhte Abundanz von Enterobacteriaceae, insbesondere Escherichia, festgestellt. Insgesamt wiesen die Ergebnisse dieser Arbeit einen starken Einfluss des Krankenhausaufenthaltes an sich auf, vor allem auf die ESBL-EC-Isolationsraten, wodurch möglicherweise Unterschiede zwischen den verschiedenen PAP-Behandlungen überdeckt wurden. Trotzdem stellen die in dieser Studie gesammelten Ergebnisse und gewonnenen Erkenntnisse einen ersten wichtigen Schritt in der Etablierung von Antibiotic Stewardship-Programmen in Pferdekliniken dar und könnten somit einen langfristigen Einfluss auf die lokale Verbreitung von ESBL-EC haben.

1. Einleitung

"Alle Dinge sind Gift, und nichts ist ohne Gift; allein die Dosis machts, dass ein Ding kein Gift sei."

Paracelsus (1493-1541)

Zu den bis heute bedeutsamen historischen Errungenschaften der Human- und Veterinärmedizin zählt die Entdeckung, industrielle Herstellung und klinische Anwendung von Antibiotika, welche nach wie vor für die Behandlung bakterieller Infektionen aller Art wie z.B. Blutstrominfektionen, Harnwegsinfekte oder Pneumonien, aber auch für die Prophylaxe von unerwünschten postoperativen Ereignissen wie bspw. Wundinfektionen, unverzichtbar sind (Eyler & Shvets, 2019; Mellinghoff et al., 2019).

In den letzten Jahren konnte ein Anstieg von Erkrankungen durch Bakterien beobachtet werden, gegen die antimikrobielle Wirkstoffe, die in der Vergangenheit häufig gegen diese Infektionserreger eingesetzt wurden, keine ausreichende Wirkung mehr zeigten. Diese Infektionserreger haben eine antimikrobielle Resistenz (AMR) entwickelt (European Centre for Disease Prevention and Control, 2022). Resistenzen gegen neue antiinfektive Wirkstoffe werden oftmals innerhalb nur weniger Jahre nach deren Markteinführung ein klinisches Problem, bei einigen Antibiotika konnte die Existenz entsprechender bakterieller Resistenzen sogar bereits vor diesem Zeitpunkt nachgewiesen werden (Kupferschmidt, 2016). Im Jahr 2014 schätzte die Weltgesundheitsorganisation (WHO) in ihrem globalen Bericht zur Antibiotika-Resistenz-Situation die weltweite verfügbare Datenlage als insgesamt schlecht ein, da u.a. in vielen Ländern Überwachungssysteme für Antibiotika-Resistenzen (auch: Surveillance) fehlten (WHO, 2014). Dabei sind Infektionskrankheiten verursacht durch resistente Mikroorganismen oftmals nur noch schwer zu behandeln, während das Risiko einer Transmission von Krankheitserregern oder schweren Krankheitsverläufen bis hin zum Tod steigt (WHO, 2021).

Eine besondere Herausforderung stellen AMR im Kontext von Hospital-assoziierten Infektionen (HAI) dar (Siegel et al., 2007). Dabei handelt es sich um Infektionskrankheiten, die von Patienten oder Mitarbeitern in einer medizinischen Einrichtung erworben werden und sich während oder nach dem Aufenthalt manifestieren (Robert Koch-Institut, 2015). HAI zählen zu den häufigsten unerwünschten Komplikationen im Gesundheitswesen und führen neben einer erhöhten Sterblichkeitsrate und verlängerten Klinikaufenthalten auch zu einer erhöhten finanziellen Belastung der Gesundheitssysteme (Sikora & Zahra, 2022). Zu den Hauptübertragungswegen für HAI-verursachende Infektionserreger zählt dabei der direkte oder indirekte Kontakt mit kontaminierten Oberflächen oder Gegenständen (Fernando et al., 2017; Sikora & Zahra, 2022). In medizinischen Einrichtungen sind zahlreiche für HAI verantwortliche Infektionserreger mit

Enterobacterales, insbesondere *Escherichia coli* (*E. coli,* auch: EC), assoziiert (Jiang et al., 2020; Singleton et al., 2021). Darüber hinaus haben Enterobacterales mit einer Resistenz gegen β-Laktam-Antibiotika in der Vergangenheit eine hohe Affinität gezeigt, zahlreiche weitere Resistenzen zu erwerben – es droht die Entstehung von Erregern, die gegen alle derzeit verfügbaren antiinfektiven Wirkstoffe resistent sind (Paterson, 2006). Eine Schätzung der weltweiten AMR-Belastung identifizierte bspw. antibiotikaresistente *E. coli* im Jahr 2019 als einen im Zusammenhang mit dem vorzeitigen Tod von Patienten am häufigsten auftretenden Infektionserreger (Murray et al., 2022).

Insgesamt beschränkt sich die Wirkung antimikrobieller Substanzen nicht nur auf die Hemmung des Wachstums oder der Vermehrung von pathogenen Mikroorganismen im Rahmen einer antiinfektiven Behandlung, sondern viel mehr erfasst sie alle Organismen der mikrobiologischen Gemeinschaft im betroffenen Habitat (Patangia et al., 2022). Diese Gesamtheit verschiedener Mikroorganismen (u.a. Viren, Pilze und Bakterien), die mit einem bestimmten Lebensraum assoziiert sind, wird als Mikrobiota bezeichnet, während die entsprechende Summe des genetischen Materials als Mikrobiom bezeichnet wird (Ursell et al., 2012).

In vielen physiologischen Prozessen, wie z.B. der Immunreaktion des Wirtes (Henao-Mejia et al., 2012), spielt die Vielfalt der Mikrobiota im Wirtsorganismus eine tragende Rolle. Zu den Folgen einer reduzierten mikrobiellen Vielfalt im enteralen Mikrobiom kann u.a. die erhöhte Anfälligkeit von Individuen für Infektionen, Autoimmunerkrankungen, Allergien, Fettleibigkeit oder Atherosklerose gehören (Becattini et al., 2016). Längst ist die Bedeutung der enteralen Mikrobiom-Vielfalt nicht vollständig verstanden, wie eine Studie zeigt, die im Zuge einer allogenen Stammzelltransplantation eine verminderte Darm-Mikrobiom-Diversität als Faktor für ein erhöhtes Sterblichkeitsrisiko für den Empfänger beschrieb (Taur et al., 2014). Die Identifikation und Interpretation von erkrankungsspezifischen Mikrobiom-Profilen ist dabei durch die hohe Individualität des Mikrobioms eines jeden Individuums eine Herausforderung (Diaz & Reese, 2021). Neben Aspekten wie Alter (Ghosh et al., 2022), Ernährung (Singh et al., 2017) oder Lebensraum (Ang et al., 2022; Tasnim et al., 2017) trägt die Transmission von pathogenen und apathogenen Mikroorganismen zwischen Individuen sowie der Umwelt maßgeblich zur individuellen Struktur des Mikrobioms bei (Benson et al., 2010; Brito et al., 2019). Eine Störung des physiologischen Gleichgewichts kann die Selektion von pathogenen Mikroorganismen im Mikrobiom sowie die Bildung eines sehr zugänglichen Reservoirs für AMR-Erreger begünstigen (Penders et al., 2013; Ramirez et al., 2020). Es erscheint demnach naheliegend, dass der weiteren Ausbreitung pathogener resistenter Bakterien, auch unter dem Aspekt der Überschneidung der für die Therapie von Krankheiten eingesetzten antimikrobiellen Wirkstoffe bei Mensch und Tier, nur mit einem „One Health“ Ansatz zu begegnen ist (McEwen & Collignon, 2018).

One Health beschreibt dabei einen kollektiven, vereinenden Ansatz, der darauf abzielt, die Gesundheit von Menschen, Tieren und Ökosystemen nachhaltig ins Gleichgewicht zu bringen und zu optimieren (One Health High Level Expert Panel (OHHLEP), 2021). Des Weiteren wird anerkannt, dass die Gesundheit von Menschen, Haus- und Wildtieren sowie Pflanzen und die weitere Umwelt (einschließlich der Ökosysteme) eng miteinander verbunden und voneinander abhängig sind (One Health High Level Expert Panel (OHHLEP), 2021). Das Verständnis der Auswirkungen der Mikrobiom-Beziehungen zwischen der Umwelt und der Gesundheit von Mensch und Tier erfordert demnach ganzheitliche und innovative Ansätze für die Diagnose und Behandlung sowie Etablierung vorbeugender Maßnahmen (Interventionen) von Krankheiten (Trinh et al., 2018).
Bereits im Jahr 2008 wurde im Zuge der Umsetzung der deutschen Antibiotika-Resistenzstrategie (DART) damit begonnen, Überwachungssysteme für Antibiotikaresistenzen und -verbrauch zu etablieren (Bundesministerium für Gesundheit, 2011). Neben dem Bedarf für eine systematische und fortlaufende Erhebung sowie Zusammenführung und Analyse von Daten (Surveillance) zu Antibiotikaresistenzprävalenzen und -inzidenzen für die Bereiche Human- und Veterinärmedizin sowie Lebensmittelproduktion und Umwelt sind geeignete übergreifende Interventionen erforderlich, um die Zusammenhänge der transsektoralen Verbreitung von Antibiotikaresistenzen besser verstehen zu können. In wie weit die Anwendung von Antibiotika in der Veterinärmedizin für die Resistenzverbreitung insgesamt eine Rolle spielt, ist bislang hauptsächlich im Hinblick auf die Nutztierhaltung untersucht, so dass der Einfluss von Heim- und Wildtieren sowie der Umwelt möglicherweise unterschätzt wird (Vercelli et al., 2022).

1.1. Antibiotika und Resistenzmechanismen

Angriffspunkte für Antibiotika in Bakterien

Verschiedene antimikrobiell wirkende Substanzen können das Wachstum und die Vermehrung von Bakterien verhindern, indem deren Stoffwechsel oder Zellteilung gestört werden. Die Wirkung dieser Substanzen lässt sich in der Folge allgemein in bakteriostatisch (eine Vermehrung wird durch die Hemmung der bakteriellen Zellaktivität unterdrückt, die einzelne Zelle stirbt aber nicht unmittelbar) und bakterizid (zelltötend) unterscheiden. Dabei werden einige für Bakterien einzigartige Strukturelemente, wie die Murein (auch Peptidoglycan) -haltige Zellwand, die Folsäuresynthese oder die spezielle Struktur der bakteriellen Ribosomen als Angriffsziel genutzt. Einen Überblick über häufig angewendete Wirkstoffgruppen und ihren zellulären Wirkort gibt Abbildung *1*.

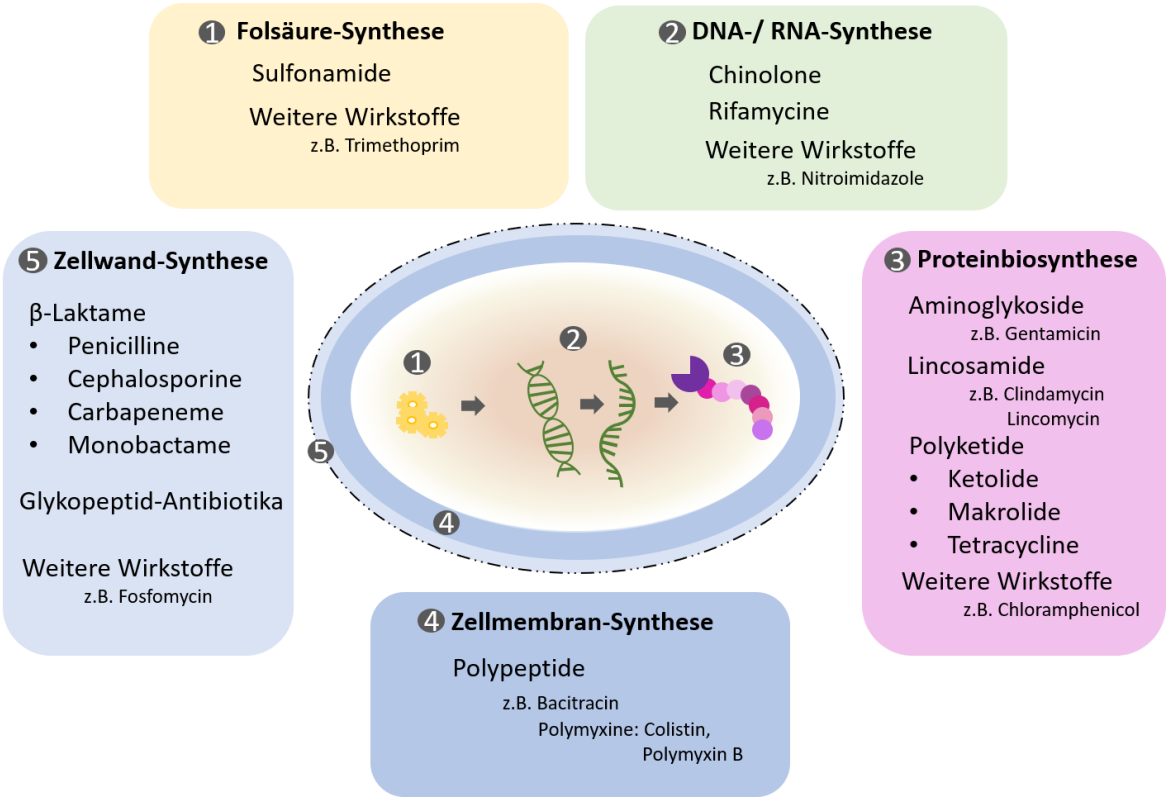

Abbildung 1| **Übersicht über gebräuchliche antibakterielle Wirkstoffe und ihren Wirkort in der Zelle**
Zu den häufigsten Angriffszielen der Antibiotika zählen die für die bakterielle Zellteilung und das Zellwachstum besonders wichtigen Stoffwechselwege wie die Folsäure-Synthese (1), die DNA-/ RNA-Synthese (2), die Proteinbiosynthese (3), die Zellmembran-Synthese (4) und die Zellwand-Synthese (5). Eine Störung dieser Stoffwechselwege führt entweder zur Hemmung der Zellteilung (bakteriostatisch) oder zum Zelltod (bakterizid). (Kauter, A. nach Pschyrembel Online (https://www.pschyrembel.de/))

Angriffsziele für Antibiotika sind u.a. die für die Zellteilung und das Zellwachstum wichtigen bakteriellen Stoffwechselwege wie die Zellwandsynthese, Proteinsynthese, Folsäuresynthese, aber auch die Stabilität und Integrität der Zellmembran. Außerdem können Antibiotika zu Strukturveränderungen an der DNA, zur Hemmung der DNA-Replikation an den Ribosomen oder zur Hemmung der DNA-abhängigen RNA-Polymerase führen und so die Zelle in ihrem Wachstum stören oder ganz hemmen. Einige Bakterien haben darüber hinaus verschiedene Strategien entwickelt, um der antimikrobiellen Wirkung dieser Agenzien zu entgehen. Bakterien der Spezies *Staphylococcus aureus* (*S. aureus*) bspw. können durch die Internalisierung in Körperzellen, wie etwa während einer Infektion, sich der Wirkung einiger Antibiotika entziehen und so überdauern (Garzoni et al., 2007). Dies ist vermutlich auch ein bedeutsamer Faktor in der Entwicklung chronischer Krankheitsverläufe (Garzoni et al., 2007). Des Weiteren kann der Verlust wichtiger Zellkomponenten, wie z.B. Thymidin unter der Anwendung von Sulfamethoxazol und Trimethoprim, von einigen *S. aureus* Spezien durch die Aufnahme der fehlenden Moleküle aus der Umgebung kompensiert werden (Goldstein & Proctor, 2008). Auch über die Synthese von Proteinen können Bakterien die Wirkung eines Antibiotikums inhibieren, wie z.B. die Wirkung von

β-Laktam Antibiotika über die Bildung von β-Laktamasen, welche viele Wirkstoffe dieser Gruppe hydrolytisch spalten können (Bush & Bradford, 2019). Darüber hinaus können Strukturänderungen von u.a. Proteinen in der Bakterienzelle dazu führen, dass in der Folge die Bindungsstellen für Antibiotika am Wirkort fehlen. Eine Vancomycinresistenz in Enterokokken wird bspw. über das Zusammenspiel von drei Genen hervorgerufen, die eine veränderte Vernetzung des Mureins über den Austausch einer Amidbindung durch eine Esterbindung zur Folge hat, wodurch die Affinität des Antibiotikums an die Zielstruktur in der Zellwand zu binden herabgesetzt wird (Selim, 2022). Darüber hinaus können spezielle Transporterproteine, z.B. das Acriflavin-Resistenz-Protein B (AcrB) aus der Familie der RND (engl. resistance nodulation-devision) Transporter in *E. coli* (Ciusa et al., 2022) dafür sorgen, dass in die Zelle eingedrungene Antibiotika wieder in den extrazellulären Raum transportiert werden und somit die Konzentration innerhalb der Zelle für eine antimikrobielle Wirkung nicht ausreichend ist (Li et al., 2015). In *E. coli* wurden bislang sechs verschiedene RND Transporterproteine identifiziert (Anes et al., 2015). Der sogenannte Target-Schutz, hierbei wird die Bindungsstelle am Zielmolekül des Antibiotikums von einem Schutzprotein besetzt und das Zielmolekül so vor einer Inhibierung bewahrt, ist ein weiterer Schutzmechanismus und kann dabei dauerhaft, durch eine induzierte chemische Veränderung am Zielmolekül, oder temporär, über eine reversible Bindung, erfolgen (Wilson et al., 2020).

Eine weitere Strategie der Bakterien ist die Bildung von sogenannten Biofilmen. Biofilme bestehen aus mikrobiellen Aggregaten, die an einer Oberfläche haften oder mit ihr verbunden sein können und in eine extrazelluläre Matrix aus polymeren Substanzen eingebettet sind (Flemming et al., 2023; Sauer et al., 2022). Diese dichte mikrobielle Akkumulation bietet einen erhöhten Schutz vor extrazellulären Stressfaktoren und vereinfacht den Austausch von genetischem Material (Flemming et al., 2023). Dabei können die verschiedenen Bestandteile der extrazellulären Matrix, z.B. Polysaccharide und negativ geladene extrazelluläre DNA, das Eindringen von antimikrobiellen Substanzen wie Antibiotika verzögern, sodass keine ausreichende Konzentration am Wirkort in der Bakterienzelle erreicht wird (Ciofu et al., 2022). Die genaue Funktionsweise der durch Biofilmbildung vermittelten Resistenz ist bis heute jedoch noch nicht vollständig erforscht (Hall & Mah, 2017).

Erbliche Unempfindlichkeiten

Einige Bakterien-Spezies zeigen eine intrinsische (chromosomal vererbliche) Resistenz, welche für alle Zellen einer Spezies, z.T. auch einer Gattung oder Familie von Bakterien, gilt. Dieses Resistenzvermögen kann sich z.B. durch das Fehlen der Zielstruktur (z.B. einer Zellwand bei Mykoplasmen) oder durch eine herabgesetzte Permeabilität der Zellwand für bestimmte Wirkstoffe zeigen, wie es auch für Mykobakterien bekannt ist (Jankute et al., 2015). Mykobakterien

besitzen aufgrund einer über dem Murein liegenden zusätzlichen Schicht aus sehr langkettigen Fettsäuren, u.a. Mykolsäuren, eine geringere Permeabilität der Zellwand im Vergleich zu anderen Bakterien. Diese Schicht führt zu einer vergleichsweise höheren Widerstandsfähigkeit gegenüber chemischen und enzymatischen Wirkstoffen und macht diese Bakterien besonders überlebensfähig (Abrahams & Besra, 2018; Jankute et al., 2015). Auch das Vorhandensein von zahlreichen Efflux-Pumpen, wie in einigen *E. coli* Stämmen (Li et al., 2015), kann eine intrinsische Resistenz gegen antimikrobielle Wirkstoffe bedingen und sollte im Falle einer antibiotischen Behandlung immer mitberücksichtig werden (Cox & Wright, 2013).

Erworbene Unempfindlichkeiten (Mutationen)

Neben intrinsischen Resistenzen können AMR auch über die Zeit erworben werden, z.B. über verschiedene genetische Veränderungen wie Mutationen oder durch die Aufnahme von mobilen genetischen Elementen (Bengtsson-Palme et al., 2018; Ghosh et al., 2020). Mutationen zeichnen sich durch eine Veränderung in der Erbinformation aus und können durch Fehler in der DNA-Replikation oder durch die Einwirkung von Mutagenen (Strahlung, Chemikalien, etc.) auf die DNA stattfinden (Watford & Warrington, 2022). Spontane Mutationen treten dabei mit einer Rate von 1 zu 10^5 bis 10^8 während der Zellteilung der Bakterien auf (Watford & Warrington, 2022). Der natürliche Prozess der Entstehung von AMR durch zufällige Mutationen kann nicht verhindert, aber in der Häufigkeit beeinflusst werden. Der unsachgemäße Gebrauch von Antiinfektiva, wie eine inadäquate Dosierung oder der Einsatz von nicht ausreichend wirksamen Antibiotika im Falle einer Infektionskrankheit, kann das Auftreten von natürlicherweise vorkommenden Mutationen beeinflussen und eine Resistenzentwicklung fördern (Durão et al., 2018; Huemer et al., 2020).

Erworbene Unempfindlichkeiten (Horizontaler Gentransfer (HGT))

β-Laktam Antibiotika inaktivieren sogenannte Penicillin-Bindeproteine (PBP) durch die Bindung an deren aktive Zentren, wodurch diese die Peptid-Quervernetzungen der Murein-haltigen Zellwandbestandteile nicht mehr ausreichend herstellen können.

Methicillin-resistente *Staphylococcus aureus* (MRSA) hingegen haben durch Aufnahme eines mobilen genetischen Elementes, der (engl.) Staphylococcal Cassette Chromosome *mec* (SCC*mec*), ein zusätzliches PBP, das sogenannte PBP2a, erworben, welches den Verlust der Quervernetzungen zwischen den Zellwandbausteinen in Gegenwart von β-Laktam Antibiotika kompensieren kann, indem es die enzymatische Aktivität des nativen PBP2 substituiert (Pinho et al., 2001). Dieser Mechanismus führt zum Erhalt der Integrität der Zellwand und zur Resistenz gegenüber vielen auf dem β-Laktam-Ring-basierenden Wirkstoffen.

Eine Resistenz gegen Tetracycline in klinisch relevanten Krankheitserregern wird vor allem über zwei Mechanismen vermittelt: aktiver Efflux des Antibiotikums sowie Protein-vermittelter Target-Schutz (Wilson et al., 2020). Die ribosomalen Schutzproteine Tet(A) und Tet(B) gehören dabei zu den am häufigsten identifizierten über HGT erworbenen Tetracyclin-Resistenzgenen in *E. coli* (Bryan et al., 2004; Karami et al., 2006; Sengeløv et al., 2003).

Klassifizierung von Bakterien mit erworbenen Resistenzen

Um die unterschiedlichen Ausprägungen von erworbener AMR besser zu klassifizieren, wurden verschiedene Definitionen aufgestellt, die das Ausmaß (den Grad) der antibakteriellen Resistenz für eine bestimmte Spezies bzw. für eine bestimmte Familie von Bakterien charakterisiert (Magiorakos et al., 2012; Schwarz et al., 2010). Unter der Voraussetzung einer eindeutigen Speziesidentifizierung des Erregers und einem Testergebnis, erhoben mittels einer standardisierten antimikrobiellen Empfindlichkeitsprüfung (z.B. nach den Richtlinien des Clinical and Laboratory Standard Institute (CLSI) oder der des European Committee on Antimicrobial Susceptibility Testing (EUCAST)), kann ein Erreger als unempfindlich, empfindlich bei erhöhter Exposition (früher: intermediär) oder empfindlich gegenüber einem antimikrobiellen Wirkstoff beschrieben werden (Gatermann et al., 2020). Darüber hinaus wurde von einem Expertenteam eine Standardterminologie vorgeschlagen, um im Gesundheitswesen häufig vorkommende pathogene Bakterien konsistent anhand ihrer Resistenzprofile in panresistente (PDR), gegen ein erweitertes Wirkspektrum (XDR) resistente und multiresistente Erreger (MRE) zu klassifizieren (Magiorakos et al., 2012). Für den im Zusammenhang mit HAI bedeutenden Erreger *E. coli* liegt nach dieser Definition eine Multiresistenz vor, wenn für mindestens einen Wirkstoff aus jeweils mindestens drei therapeutisch relevanten Wirkstoffklassen eine Unempfindlichkeit nachgewiesen werden kann (Magiorakos et al., 2012). MRE *E. coli* können sich scheinbar besonders gut an diverse Habitate anpassen, wie z.B. an verschiedene Wirte und Umweltbedingungen, gegebenenfalls Reservoire bilden und in der Folge zwischen verschiedenen Sektoren übertragen werden (EFSA & ECDC, 2020; Mathers et al., 2015).

1.1.1. Resistenz durch Produktion von β-Laktamasen

Eine in der Human- und Veterinärmedizin weit verbreitete und klinisch bedeutende Resistenz ist die verminderte Empfindlichkeit von Infektionserregern gegen β-Laktam-Antibiotika, denn diese Wirkstoffgruppe gehört aufgrund ihrer breiten Wirksamkeit und allgemein guten Verträglichkeit zu den häufigsten therapeutisch eingesetzten Antibiotika überhaupt (Bush & Bradford, 2016; Klein

et al., 2018; WHO, 2018). β-Laktam-Antibiotika hemmen die Zellwandsynthese der Bakterien, indem sie als Substratanaloga im aktiven Zentrum der Transpeptidasen (die PBP) an das dort zentral positionierte Serin irreversibel binden (Bush & Bradford, 2016). Durch diesen Eingriff in den zentralen Prozess des Zellwandaufbaus wird eine mangelhafte Zellwand ausgebildet, die die Integrität der Zelle nicht mehr gewährleiten kann. In der Folge wird das osmotische Gleichgewicht der Zellen im Wachstum gestört, bis diese schließlich anschwellen und platzen. Bei einer auf der Bildung eines bakteriellen Enzyms (β-Laktamase) beruhenden β-Laktam-Resistenz wird der β-Laktam-Ring des Antibiotikums gespalten und damit die Wirkung inhibiert (Poole, 2004). Durch Punktmutationen in den für β-Laktamasen-kodierenden Genen können diese Hydrolasen ihr Wirkspektrum verändern und erweitern, man spricht von einem Enzym mit erweitertem Wirkungsspektrum, (engl.) Extended Spectrum β-Lactamase (ESBL) (Bradford, 2001). Die genaue Definition der ESBLs folgt bisher keinem gemeinsamen Konsens, als allgemeine Definition kann jedoch eine bakterielle Resistenz gegen Penicilline, Cephalosporine (erste, zweite und dritte Generation) und Aztreonam jedoch keine Resistenz gegen Cephamycine oder Carbapeneme angenommen werden (Lee et al., 2012; Paterson & Bonomo, 2005). Außerdem können ESBLs durch β-Laktamase-Inhibitoren wie Clavulansäure gehemmt werden (CLSI, 2020; Paterson & Bonomo, 2005). Da die für ESBL-kodierenden Gene häufig auf Plasmiden lokalisiert sind, können sie über HGT weitergegeben werden. Nicht selten sind auf entsprechenden Plasmiden weitere Resistenzen gegen andere Antibiotika lokalisiert (Li et al., 2019). Neben den hier beschriebenen ESBL-Enzymen können auch eng verwandte Cephamycinasen (AmpC-β-Laktamasen) von einigen Bakterien gebildet werden. AmpC-β-Laktamasen vermitteln ebenfalls eine Resistenz gegen Penicilline und Cephalosporine (außer: 4. Generation), aber auch gegen Cephamycine. Des Weiteren zeigen sie eine phänotypische Resistenz gegenüber Laktamase-Inhibitoren, weshalb es in Anwesenheit von AmpC-β-Laktamasen bei ESBL-Bestätigungstests zur Überlagerung von verschiedenen Resistenzenzphänotypen kommen kann und eine gesonderte diagnostische Überprüfung notwendig ist (siehe auch Kapitel 1.1.2).

Die Klassifizierung der verschiedenen β-Laktamasen erfolgt nach keinem Goldstandard. Zur Charakterisierung kann die Aminosäuresequenz (Ambler-Klassifikation A bis D) (Ambler et al., 1980) herangezogen oder eine Einteilung nach Substrat und Inhibitor (Bush und Jacoby Klassifikation) (Bush & Jacoby, 2010) vorgenommen werden.

In der Datenbank des National Center for Biotechnology Information (NCBI) sind derzeit (Februar 2023) ca. 537 ESBL-Referenzgene der β-Laktamase-Familien BEL, CTX-M, GES, OXY, PER, SHV, TEM, TLA und VEB (Castanheira et al., 2021; Jacoby, 2006) gelistet (National Center for Biotechnology Information, 2004). Häufig sind diese Gene bei Enterobacterales zu finden, wie bspw. *E. coli*, *Klebsiella pneumonia (K. pneumonia)*, *Klebsiella oxytoca* (*K. oxytoca*) oder *Enterobacter cloacae*.

1.1.2. Nachweis von ESBL-produzierenden Enterobacterales in Proben von Pferden

Die Verbreitung von zoonotischen und multiresistenten Erregern, wie z.B. ESBL-produzierende Enterobacterales, sind eine ständige Herausforderung für die Sicherheit und Hygiene innerhalb von veterinärmedizinischen Einrichtungen (Royden et al., 2019; Walther et al., 2018; Walther et al., 2017). Speziell innerhalb von Pferdekliniken konnten Studien eine kontinuierliche Verbreitung von ESBL-produzierenden *E. coli* (ESBL-EC) unter den stationär aufgenommenen Pferdepatienten zeigen (Apostolakos et al., 2017; Walther et al., 2018). Darüber hinaus konnten HAI mit einer lokalen Ausbreitung von MRE in Pferdekliniken in Verbindung gebracht werden (van Spijk et al., 2019; Walther et al., 2014). Diese Infektionen sind therapeutisch oft schwierig zu handhaben, da eine Vielzahl zusätzlicher AMR häufig mit ESBL-EC assoziiert sind (Wieler et al., 2011)

Das Screening und der Nachweis von ESBL-produzierenden klinisch bedeutsamen gram-negativen Infektionserregern erfolgt über verschiedene phänotypische Charakterisierungen. Für die Definition der ESBL-Eigenschaften und Auswertungskriterien für Testergebnisse aus phänotypischen Verfahren gibt es festgesetzte Konventionen, z.B. in den Interpretationsvorschriften des CLSI (siehe auch Tabelle *1*) (CLSI, 2020). Einen ersten Hinweis auf eine ESBL-Aktivität können ESBL-Screenings geben. Eine häufig genutzte Methode ist dabei der Agardiffusionstest mittels definierter Mengen bestimmter Antibiotika oder auch ein Screening mittels Dilutionsmethoden zur Bestimmung der minimalen Hemmkonzentrationen (MHK).

In einer zweiten Stufe, die Bestätigung der ESBL-Bildung, werden insbesondere Ceftazidim und Cefotaxim in Ab- und Anwesenheit von Clavulansäure über das Agardiffusions- oder das Mikrodilutionsverfahren getestet (CLSI, 2020). Clavulansäure fungiert dabei als β-Laktamase-Inhibitor und führt im Falle einer β-Laktamase-Produktion trotz dessen Aktivität zu einer Hemmung des Wachstums der Bakterien (Kim et al., 2009). Die Testergebnisse werden dann mit den in den jeweiligen Normen festgelegten Grenzwerten für die getesteten Antibiotika/ Inhibitoren Kombinationen verglichen und so die ESBL-Bildung für ein Isolat bestätigt (CLSI, 2020). Bei den durchgeführten Nachweisen muss immer mit der möglichen Anwesenheit von weiteren β-Laktamasen, bspw. mit AmpC-β-Laktamasen (chromosomal oder erworben) gerechnet werden, da sie durch ihre Eigenschaften das phänotypische Resistenzbild von der reinen ESBL-Produktion überlagern können. Aus diesem Grund ist der Nachweis der ESBL-Bildung zwingend an eine eindeutige vorherige Speziesidentifikation gebunden (Adel et al., 2022). Für die im Zusammenhang mit HAI häufig auftretenden Pathogene *K. pneumoniae*, *K. oxytoca* und *E. coli* sind die nach CLSI festgelegten Normen in Tabelle *1* zusammengefasst.

Tabelle 1| **Screening und Nachweis-Kriterien für ESBL in *K. pneumonia*, *K. oxytoca* und *E. coli* nach CLSI Konventionen.** ESBL Screening: Hemmzonen und -konzentrationen über den angegebenen Grenzwerten weisen auf eine ESBL Aktivität hin. ESBL Nachweis: Für den Agardiffusionstest muss in mindestens einer Kombination (Wirkstoff mit und ohne Clavulansäure) ein um mindestens 5 mm vergrößerter Hemmhof in Kombination mit Clavulansäure gezeigt werden. Im Nachweis per Mikrodilutionsverfahren muss für jedes in Kombination mit Clavulansäure getestete Antibiotikum eine dreifache Abnahme der minimalen Hemmkonzentration im Vergleich zur Testung des Antibiotikums ohne Clavulansäure gezeigt werden. Konz. = Konzentration.

ESBL Screening				
	Agardiffusion		*Mikrodilution*	
Wirkstoff	**Konzentration in µg**	**Hemmhofgrenze in mm**	**Konzentration in µg/mL**	**MHK in µg/mL**
Cefpodoxim	10	≤17	4	≥8
Ceftazidim	30	≤22	1	≥2
Aztreonam	30	≤27	1	≥2
Cefotaxim	30	≤27	1	≥2
Ceftriaxon	30	≤25	1	≥2
ESBL Nachweis				
	Agardiffusion		*Mikrodilution*	
Wirkstoff	**Konzentration in µg**	**Hemmhofgrenze in mm**	**Konzentration in µg/mL**	**MHK in µg/mL**
Ceftazidim	30		0,25 - 128	
Ceftazidim/ Clavulansäure	30 / 10	+ ≥5	0,25/ 4 - 128/ 4	- ≥3-fache Konz.
Cefotaxim	30		0,25 - 64	
Cefotaxim/ Clavulansäure	30 / 10	+ ≥5	0,25/ 4 - 64/ 4	- ≥3-fache Konz.

(CLSI, 2020)

Um diese Routinetests im Labor zu vereinfachen, wurden außerdem kommerzielle, automatisierte Systeme auf Basis der Mikrodilution entwickelt. Diese Systeme ermitteln über eine zeitaufgelöste Messung der optischen Dichte[1] (OD_{600}) der Inokulum-Suspensionen die entsprechenden MHK-Werte.

Eine exakte Identifizierung von ESBL-Genen kann mit den hier beschriebenen Methoden jedoch nicht erfolgen, sondern nur über molekulare Analysen realisiert werden. In der routinemäßigen Diagnostik ist die Amplifikation mittels PCR und Sequenzierung der einzelnen ESBL-Gene häufig mit einem großen Arbeitsaufwand und hohen Kosten verbunden und wird daher nur in ausgewählten Fällen umgesetzt.

[1] mittels Photometer gemessene Extinktion von Lösungen, u.a. zur Ermittlung der Zelldichte

1.1.3. Methoden zur Untersuchung und Analyse des Mikrobioms aus Proben von Pferden

Die Untersuchung der Zusammensetzung des Mikrobioms, auch das von Pferden, kann derzeit durch verschiedene Technologien realisiert werden (*Abbildung 2*).

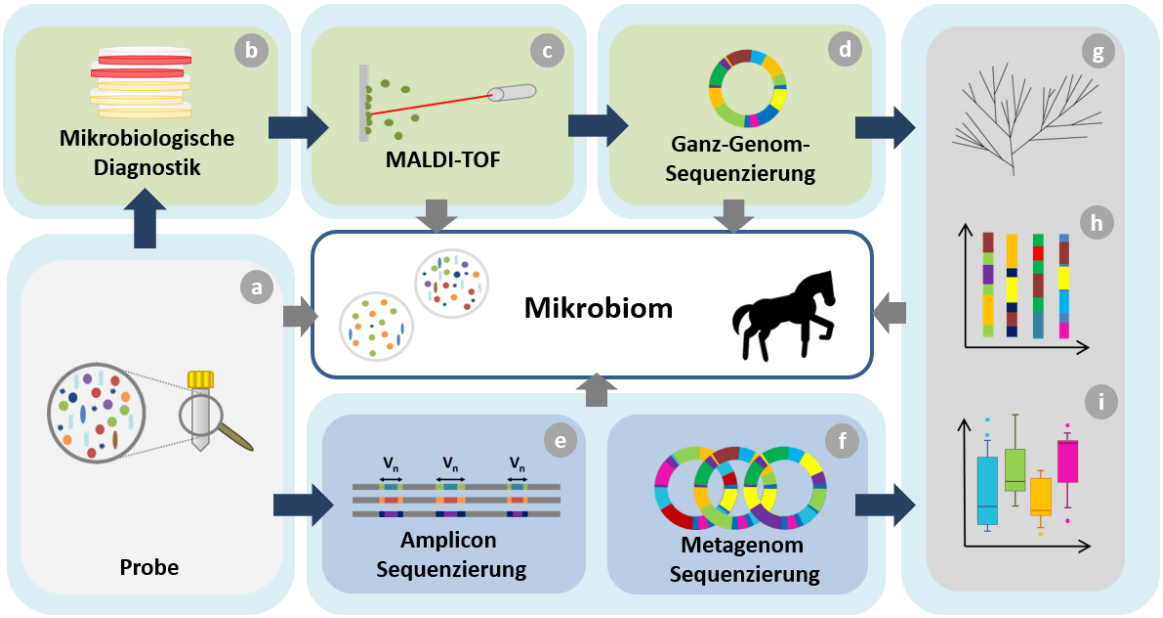

Abbildung 2| **Integrativer und synergistischer Arbeitsablauf zur Untersuchung des Mikrobioms von Pferden**. Aufteilung der frischen Probe (a) für zwei verschiedene Untersuchungsansätze: mikrobielle Diagnostik (b-d) und DNA-Sequenzierung (e und f) für die Populationsanalyse (g-i). Für die mikrobielle Diagnostik (b) wird ein breites Spektrum unterschiedlicher aerober und anaerober Kulturbedingungen verwendet, gefolgt von einer Speziesidentifikation durch (engl.) Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (kurz: MALDI-TOF-MS) (c). Die Genomsequenzierung (d) ermöglicht die Identifizierung von (neuen) Arten sowie die Identifikation von Resistenz-kodierenden Genen oder anderen Faktoren.
Die DNA-Extraktion für die Sequenzierung der variablen Regionen des 16S/18S rRNA-Gens (e) ermöglicht die Charakterisierung und Quantifizierung der taxonomischen Einheiten innerhalb der Probe oder auch die Identifizierung aller in einer Probe vorhandenen Genome (Metagenom) (f). Weitere bioinformatische Verfahren umfassen u.a. die Beschreibung der Phylogenetik (g), relative taxonomische Häufigkeiten (h) sowie Diversitätsindizes (i). Die Kombination von klassischer Diagnostik und verschiedenen Techniken zur Erzeugung von Genomdaten ermöglicht tiefe Einblicke in die Zusammensetzung und die Merkmale des Mikrobioms. (Kauter, A., adaptiert von Abbildung 1 aus **Veröffentlichung I**).

Die Identifizierung von Darmmikroorganismen wurde noch bis vor wenigen Jahren vor allem mit kulturabhängigen Methoden durchgeführt, deren Ergebnisse sich folglich auf kultivierbare Spezies beschränkten (Panek et al., 2018). Diese Methoden werden jedoch allmählich durch neue und umfassendere Ansätze wie "Culturomics" ersetzt und ergänzt. „Culturomics“ beschreibt dabei eine Form der Probenprozessierung, bei der verschiedene Wachstumsbedingungen und Inkubationszeiten genutzt werden, um möglichst viele Mikroorganismen zu kultivieren. Die Kombination mit schnellen Identifizierungsmethoden für Bakterien (z.B. Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF-MS)), aber auch der

zusätzliche Einsatz von 16S Gen rRNA-Sequenzierungen (Lagier et al., 2018) ermöglicht nun die zeitnahe nähere Untersuchung von bisher nicht kultivierbaren Spezies (Lagier et al., 2018).
Neue und moderne DNA-Sequenzierungstechnologien ((engl.) next generation sequencing (NGS)) ermöglichen darüber hinaus einen hohen Durchsatz in der Untersuchung komplexer biologischer Proben auf der Grundlage von Sequenzinformationen (Quince et al., 2017) und gehören zu den vorherrschenden Methoden in der derzeitigen Mikrobiomforschung. Je nach Sequenziermethode kann eine unterschiedliche Tiefe an Informationen gewonnen werden. Im Allgemeinen kann nach der Aufreinigung der DNA aus den Proben und einer nachfolgenden Amplicon-Sequenzierung eines ubiquitären Markergens, wie bspw. das 16S rRNA-Gen für Bakterien, das 18S rRNA-Gen für Eukaryonten oder eine interne transkribierte Spacer-DNA zwischen den rRNA-Genen für Pilze, (Kim et al., 2017; Wensel et al., 2022) eine Charakterisierung der zugehörigen Taxa erfolgen. Die Ganz-Genom-Shotgun-Sequenzierung wiederum ermöglicht über eine zufällige Fragmentierung der DNA und der anschließenden Sequenzierung der Fragmente die Rekonstruktion der gesamten ursprünglich zugrunde liegenden DNA, wobei besonders lange DNA-Fragmente und die Überlappung von Fragmenten die Genauigkeit erhöhen (Ranjan et al., 2016). Neben einer taxonomischen Einordnung können so auch vorhandene Gene (z.B. Resistenzgene) identifiziert werden.

1.2. Zielstellung

Wie oben aufgezeigt, stellen Veterinärkliniken ein Zentrum für die Akkumulation und Verbreitung mehrfachresistenter Indikatorpathogene dar. Im Zusammenhang mit HAI und dem Auftreten von zoonotischen und multiresistenten Erregern in der Veterinärmedizin sind vor allem MRSA, ESBL-EC und *Acinetobacter baumannii* seit vielen Jahren bekannt, die aktuellen Fortschritte bei der praktischen Umsetzung von Infektionskontrollprogrammen in Tierkliniken sind jedoch begrenzt (Walther et al., 2018; Walther et al., 2017). Durch die Entwicklung und Prüfung von Antibiotika-Stewardship-Programmen (McEwen & Collignon, 2018) unter Berücksichtigung des One Health Ansatzes wird eine Verbesserung hinsichtlich Eindämmung der Akkumulation und Verbreitung mehrfachresistenter Indikatorpathogene in diesem Bereich angestrebt. Die hier vorgestellten Studienergebnisse sind Teil eines vom Bundesministerium für Bildung und Forschung geförderten interdisziplinären Forschungsnetzwerks (#1HealthPREVENT), welches u.a. Strategien zur Verbesserung des sorgfältigen Einsatzes von Antibiotika in der Veterinärmedizin erarbeitet.

Die Ziele dieser Arbeit sind:

i) Darstellung des gegenwärtigen Forschungs- und Wissensstands zur enteralen Mikrobiota, zum Mikrobiom und Metagenom von Pferden; Konzeption eines integrierten Ansatzes zur synergistischen Nutzung verschiedener Untersuchungstechniken („Culturomics“, Empfindlichkeitsprüfung von Indikatorpathogenen sowie Multiomics-Analysen) zur Darstellung von Antibiotika-induzierten Veränderungen im equinen Darmtrakt (**Veröffentlichung I**)

ii) Evaluierung des Einflusses von unterschiedlichen peri-operativen Antibiotika-Regimen ((engl.) perioperative antibiotic prophylaxis, kurz: PAP) auf die Isolationsrate von ESBL-EC bei aufgrund von Kolik hospitalisierten Pferden (**Veröffentlichung II**)

iii) Charakterisierung der Diversität und Dynamiken des enteralen Mikrobioms der in ii) erfassten Proben von Pferden (**Veröffentlichung III**)

2. Zusammenfassung und Diskussion der Ergebnisse

2.1. **Veröffentlichung I**

Kauter A, Epping L, Semmler T, Antao EM, Kannapin D, Stoeckle SD, Gehlen H, Lübke-Becker A, Günther S, Wieler LH, Walther B. The gut microbiome of horses: current research on equine enteral microbiota and future perspectives. Anim Microbiome. 2019 Nov 13;1(1):14. doi: 10.1186/s42523-019-0013-3.

Sowohl in der Human- wie auch in der Veterinärmedizin sind die komplexen Wechselwirkungen innerhalb mikrobieller Gemeinschaften, einschließlich Bakterien, Archaeen, Parasiten, Viren und Pilze, die mit einer beeinträchtigten Gesundheit oder Krankheiten einhergehen, ein Forschungsgebiet von zunehmender Bedeutung. Erkrankungen des Magen-Darm-Trakts und deren Folgen gehören zu den behandlungsintensivsten Krankheiten bei domestizierten Equiden. Das derzeitige Wissen zu den Veränderungen des Mikrobions im Verlauf von enteralen Erkrankungen ist jedoch begrenzt und die auf das Mikrobiom abzielenden Interventionen insgesamt rar. Derzeitig verfügbare Literatur (2023) über das equine enterale Mikrobiom spiegelt ein großes Ungleichgewicht wieder, da nur wenige Studien Archaeen, Viren und Eukaryoten berücksichtigen, im Vergleich zu den verfügbaren Daten über bakterielle Komponenten (**Veröffentlichung I**).

Bis vor kurzem wurden für die Identifizierung und Beschreibung der Veränderungen in der Zusammensetzung der enteralen Mikroorganismen kulturabhängige Methoden verwendet, die das Ergebnis nur auf die im Labor kultivierbaren Bakterien beschränkten. Neue umfassende Ansätze wie "Culturomics" bieten einen schnellen und umfassenden Überblick über die kultivierbaren bakteriellen Bestandteile einer Probe, da hier eine Vielzahl unterschiedlicher Nährmedien eingesetzt wird. Zusammen mit den verfügbaren Sequenzierungstechnologien, welche die Gesamtheit der Gene (Mikrobiom), inkl. die der nicht-kultivierbaren Mikrobiota, abbilden können, sind komplexe biologische Proben in hoher Auflösung analysierbar (siehe auch Kapitel 1.1.3.).

Zu den wichtigsten Zielen in der Mikrobiom-Forschung gehört die Beschreibung von Unterschieden in den relativen Häufigkeiten von Bakterientaxa, die durch Umweltveränderungen oder andere definierte Einflüsse, wie z.B. eine Antibiotika-Behandlung, verursacht werden. Um die Variation und Zusammensetzung mikrobieller Gemeinschaften zu messen und zu analysieren, wurden verschiedene Indizes zur Beschreibung der Diversität eingeführt. Im Allgemeinen wird dabei die Alpha-Diversität als eine Schätzung der Artenzahl (Reichtum) und der Verteilung (Gleichmäßigkeit) innerhalb einer bestimmten Probe beschrieben. Die Beta-Diversität dient als ein Ähnlichkeitsmaß für (Gesamt-)Populationen zwischen verschiedenen Proben (Andermann et al., 2022). Eine stetig

wachsende Anzahl an bioinformatischen Tools liefert dabei die Möglichkeit verschiedene Ansätze anhand eines Datensatzes zu erschließen, wobei zu den am häufigsten angewendeten Diversitätsmaßstäben der Shannon-Index (Spellerberg & Fedor, 2003) bezogen auf die (engl.) operational taxonomic units (OTUs) (Blaxter et al., 2005) einer Probe gehört.

2.1.1. Mikrobiom des Pferdes

Pferde sind ausschließliche Pflanzenfresser und gehören zur Gruppe der Enddarmfermentierer. Das intestinale Mikrobiom dieser Tiere wird geprägt von Bacteroidota sowie zellulose- und xylanverarbeitenden Bakterien (Dougal et al., 2014), aber auch von Pilzen, Protozoen, Archaeen, Viren und anderen Bakterien (Costa & Weese, 2018; **Veröffentlichung I**). Jedes Kompartiment innerhalb des equinen Verdauungstraktes zeigt eine individuelle Gemeinschaft von Mikroorganismen. Benachbarte Kompartimente sind dabei ähnlicher in ihrer Zusammensetzung als weit voneinander entfernte Abschnitte des Verdauungstraktes (Costa et al., 2015; **Veröffentlichung I**). Im Allgemeinen lässt sich der Gastrointestinaltrakt (GIT) von Pferden jedoch in zwei Regionen unterteilen: eine obere und eine untere Region (Ericsson et al., 2016). Die Zusammensetzung des Mikrobioms des oberen Verdauungstrakts (Magen, Duodenum, Jejunum und Ileum) ist dabei durch die Aufnahme von Umweltbakterien aus Futter/ Wasser/ Luft beeinflusst und daher variabler. Dieser GIT-Abschnitt ist vor allem durch α-Proteobacteria wie *Methylobacterium* spp., *Rhizobium* spp. und *Sphingomona*s spp. geprägt (Ericsson et al., 2016). Im Gegensatz dazu ist das Mikrobiom des unteren Verdauungsabschnitts (Zäkum und Kolon) relativ stabil in seiner Zusammensetzung, abgesehen von individuellen Einflüssen wie Alter, Geschlecht oder Rasse des Pferdes (**Veröffentlichung I**). Neugeborene Fohlen zeigen in der Regel eine variable Zusammensetzung der enteralen Mikrobiota, welche sich bis zu einem Alter von ungefähr 9 Monaten stabilisiert und sich danach kaum noch von der der erwachsenen Individuen unterscheidet (Costa et al., 2016).

Die Eigenschaften und Wechselwirkungen der häufig in einem bestimmten Kompartiment bzw. Lebensraum vorkommenden Mikroorganismen sind dabei wahrscheinlich ein wichtiger Bestandteil der Integrität und Funktion dieses Kompartiments (Shade & Handelsman, 2012). Die Definition einer essentiellen Gemeinschaft an Mikroorganismen („Kernmikrobiota“) kann demnach nützlich sein, um die Auswirkungen von Ungleichgewichten vorherzusagen und die mit einem gesunden Zustand assoziierte Gemeinschaft zu erhalten oder wiederherzustellen (Shade & Handelsman, 2012; **Veröffentlichung I**).

Eine Vielzahl von Faktoren hat Einfluss auf das Gleichgewicht der Mikroorganismen im equinen Verdauungssystem. Dazu gehören Faktoren wie Bewegung (Górniak et al., 2021), Stress durch

Transporte (Schoster et al., 2016), Ernährung (Hesta & Costa, 2021) oder die Verabreichung von Antibiotika (Barr et al., 2013). Ein Ungleichgewicht der Mikrobiota im Darm kann u.a. Kolik und Colitis bei Pferden verursachen (Costa et al., 2012). Zusammenfassend lässt sich sagen, dass die gesamte Diversität der bakteriellen Gemeinschaft von domestizierten Pferden erstaunlich gering zu sein scheint, eine Tatsache, die als möglicher Grund für die Anfälligkeit von Pferden für GIT-Erkrankungen diskutiert wurde (Dougal et al., 2014; **Veröffentlichung I**).

2.1.2. Konzeption des Studiendesigns und Studienablauf

Basierend auf den zusammengetragenen Erkenntnissen der vorangestellten Veröffentlichung (**Veröffentlichung I**) wurde das Studiendesign für die zwei weiteren Fragestellungen dieser Arbeit entwickelt (Abbildung *3*).

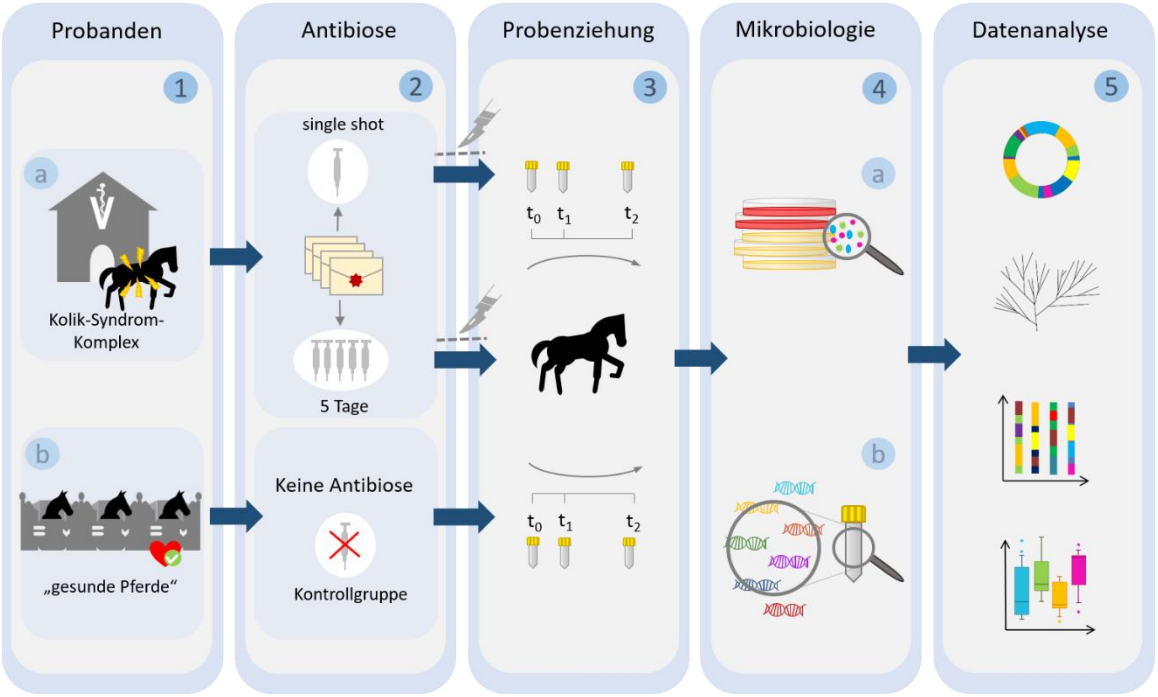

Abbildung 3|**Zusammenfassende Darstellung des Studiendesigns.** Aufgrund eines Kolik-Syndroms hospitalisierte Pferde (1a) werden per Los einem von zwei PAP-Regimen zugeteilt (2). Von allen Tieren wurden 2x Nüstern- sowie Kotproben (Set A, Set B) am Tag der Ankunft (t_0) sowie drei (t_1) und zehn (t_2) Tage postoperativ genommen (3). Alle (A) Proben wurden jeweils einem mikrobiologischen Screening auf ESBL-EC unterzogen (4a). Zur Analyse des Mikrobioms wurden alle (B) Proben außerdem einer 16S rRNA-Gen Sequenzierung unterzogen (4b). Anschließend wurden die Ergebnisse (4) aller Untersuchungen auf den Einfluss der zwei verschiedenen PAP-Regime hin ausgewertet (5). Analog zur Untersuchung der hospitalisierten Pferde wurden klinisch gesunde Pferde einer Stallgemeinschaft (1b) als Kontrollgruppe zur Studie hinzugezogen. Die Beprobung der Tiere wurde entsprechend am Starttag (t_0) sowie nach drei (t_1) und zehn Tagen (t_2) durchgeführt. Die Analyse der Proben der Kontrollgruppe erfolgte analog zur Gruppe der hospitalisierten Tiere. (**Veröffentlichungen II und III**)

Im Rahmen der hier vorgestellten Studie zur Verbesserung des sorgfältigen Einsatzes von Antibiotika in der Veterinärmedizin und speziell beim Pferd, wurden zwei verschiedene PAP-

Regime („single shot“ Gruppe (SSG) versus „5 days“ Gruppe (5DG)) evaluiert, welche bei einem abdominalen chirurgischen Eingriff im Zusammenhang mit einem diagnostizierten Syndrom-Komplex „Kolik“ zum Einsatz kamen. Die in die Studie einbezogenen Pferde wurden per Los jeweils einer der zwei Gruppen zugeordnet und erhielten eine Kombination aus Penicillin und Gentamicin (kurz: P/G) (siehe auch (Stöckle et al., 2021)). Zusätzlich zur Gruppe der hospitalisierten Pferde wurden auch Tiere einer Stallgemeinschaft ohne klinische Auffälligkeiten als nicht-hospitalisierte Kontrollgruppe einbezogen (**Veröffentlichungen II und III**).

Zur Untersuchung der ESBL-EC Last und des Einflusses der zwei verschiedenen Antibiotika-Regime auf das Mikrobiom wurden die Kotproben und Nüsternabstriche der Tiere an drei verschiedenen Zeitpunkten (Klinikaufnahme (t_0), Tag 3 (t_1) und Tag 10 (t_2) postoperativ) untersucht (Abbildung *3*). Analog zu t_0 und t_1 wurden auch die Proben der Kontrollgruppe prozessiert. Alle Proben wurden hinsichtlich ESBL-EC untersucht und die identifizierten Isolate phänotypisch (ESBL-Nachweis mittels Agardiffusionstest, MHK -Bestimmung mittels automatisierter Antibiotika-Empfindlichkeitsprüfung im Vitek-System) (Institut für Mikrobiologie und Tierseuchen, Freie Universität Berlin) und genotypisch (Ganzgenom-Sequenzierung) charakterisiert (**Veröffentlichung II**). Außerdem wurden die Proben (Set B) einer 16S rRNA-Gen Sequenzierung der V1-V2 Region unterzogen (durchgeführt am Institut für Klinische Molekularbiologie der Christian-Albrechts-Universität zu Kiel). Anschließend erfolgte die bioinformatische Aufbereitung (Silver Anthony Wolf, Abteilung MFI, Robert Koch-Institut) sowie die Fach-übergreifende Analyse und Interpretation der gewonnenen Daten (**Veröffentlichung III**).

2.2. Veröffentlichung II

Kauter A, Epping L, Ghazisaeedi F, Lübke-Becker A, Wolf SA, Kannapin D, Stoeckle SD, Semmler T, Günther S, Gehlen H, Walther B. Frequency, Local Dynamics, and Genomic Characteristics of ESBL-Producing Escherichia coli Isolated From Specimens of Hospitalized Horses. Front Microbiol. 2021 Apr 16;12:671676. doi: 10.3389/fmicb.2021.671676.

Tierkliniken gelten als Hotspots für die Akkumulation und Ausbreitung von multiresistenten ESBL-EC (Royden et al., 2019; Walther et al., 2017). Darüber hinaus wird der Kontakt mit Pferden immer wieder als ein möglicher Risikofaktor für die Übertragbarkeit von ESBL-EC auf den Menschen diskutiert (de Lagarde et al., 2019; Hordijk et al., 2020; Huijbers et al., 2013). Der Klinikaufenthalt an sich sowie die Verabreichung von Antibiotika wurden erst kürzlich als wichtige Risikofaktoren für die Kolonisierung der Tiere mit multiresistenten EC bzw. ESBL-EC identifiziert (Ahmed et al., 2012; Schoster et al., 2020). Studien zu ESBL-EC-Isolationsraten und lokalen Dynamiken dieser Indikatorpathogene innerhalb bestimmter Patientengruppen, z.B. bei aufgrund von Kolik laparatomierten und hospitalisierten Pferden, sind bislang rar.

Bei nicht-hospitalisierten Pferden konnte die Besiedlungsrate für ESBL-Enterobacterales mit einer vorangegangenen antibiotischen Behandlung oder tierärztlichen Untersuchung in Zusammenhang gebracht werden (Kaspar et al., 2019). Dagegen zeigte eine Studie an verwilderten geografisch isolierten Pferden durchweg eine Empfindlichkeit gegen die getesteten antimikrobiellen Wirkstoffe für fast alle (97%) der in Proben von diesen Tieren identifizierten EC-Isolate (Timonin et al., 2017). Der umsichtige Einsatz von Antibiotika zur Verringerung des Selektionsdrucks, speziell im klinischen Umfeld, scheint daher ein wichtiger Schritt zu sein, um die Biosicherheit für Tierpatienten und Krankenhauspersonal zu verbessern. Diesem Ansatz folgend wurden in dieser Veröffentlichung die Auswirkungen unterschiedlicher PAP-Regime (SSG versus 5DG) auf die Häufigkeit von ESBL-EC bei hospitalisierten Pferden binnen 10 Tagen nach einer Kolik-Operation untersucht. Insgesamt erfüllten 81 von 98 hospitalisierten und beprobten Pferden die Einschlusskriterien (alle Kriterien in **Veröffentlichung II**) für diesen Teil der Studie.

2.2.1. Einfluss der PAP auf ESBL-EC Isolationsraten

Die beobachteten ESBL-EC Raten lagen für die bei der Ankunft in der Klinik untersuchten Kotproben zwischen 5 % (SSG) und 10 % (5DG) (**Veröffentlichung II**). Diese Nachweisraten stimmen im Allgemeinen mit früheren Studien überein, die eine Besiedlungsrate von 7 bis 16 % aufzeigten (Maddox et al., 2012; Schoster et al., 2020; Walther et al., 2018; **Veröffentlichung II**) Die ESBL-EC-Nachweisrate in den untersuchten Proben stieg innerhalb von drei Tagen (t_1) nach

der Klinikaufnahme von 5% auf 37% (SSG) bzw. von 10% auf 47% (5DG) an. Damit ergab sich unabhängig vom P/G-PAP-Schema für die Pferde eine 9,12-mal (95% CI 2,79-29,7) höhere Wahrscheinlichkeit ESBL-EC positiv an t_1 zu sein im Vergleich zum Zeitpunkt der Ankunft (t_0) (**Veröffentlichung II**). Der Einfluss unterschiedlich andauernder PAP auf die ESBL-EC-Raten hospitalisierter Pferde war bereits Teil einer 2020 veröffentlichten Studie (Schoster et al., 2020). Die Kotproben innerhalb dieser Untersuchung wurden u.a. bei der Aufnahme ins Krankenhaus, sowie 48-60 Stunden danach entnommen (Schoster et al., 2020). Die Autoren konnten überraschenderweise keinen signifikanten Unterschied hinsichtlich der ESBL-EC-Ausscheidungsraten zwischen Pferden, welche ein Cephalosporin der vierten Generation oder die übliche P/G-Kombination erhalten hatten, feststellen (Schoster et al., 2020). Obwohl weder das Studiendesign noch die Studienpopulation dieser Studie direkt mit der hier vorliegenden Arbeit (**Veröffentlichung II**) vergleichbar sind, sind dennoch interessante Ähnlichkeiten erkennbar. Unter anderem berichten Schoster et al., dass die ESBL-EC-Isolationsraten von Pferden, welche überhaupt keine Antibiotika erhielten, innerhalb von drei Tagen nach der Aufnahme von 6,9 % auf 36 % anstiegen (Schoster et al., 2020; **Veröffentlichung II**). Folglich scheint ein direkter und enormer Einfluss des Klinikaufenthalts auf die ESBL-EC Nachweisrate der Pferdepatienten-Proben wahrscheinlich zu sein (**Veröffentlichung II**). Es ist anzunehmen, dass auch die Auswirkungen der unterschiedlichen P/G-PAP-Regime in der hier vorliegenden Arbeit durch diese Effekte überlagert wurden, da die ESBL-EC-Nachweisrate in den Proben der hospitalisierten Pferde innerhalb von zehn Tagen nach Klinikaufnahme für beide PAP-Regime auf ein ähnliches Maß (SSG t_2 = 56%; 5DG t_2 = 57%) anstieg (**Veröffentlichung II**). An t_2 lag somit die Wahrscheinlichkeit im Vergleich zum Zeitpunkt der Ankunft für die Tiere 15,64-mal (95% CI 4,57-53,55) höher ESBL-EC positiv zu sein. Da jedoch die ESBL-EC-Nachweisrate in den aus der SSG stammenden Kotproben an t_1 insgesamt niedriger war, als bei denen der 5DG, scheint auch eine positive Auswirkung des geringeren Selektionsdrucks im Zusammenhang mit dem kurzfristigen P/G PAP-Regime wahrscheinlich zu sein. Aufgrund der begrenzten Anzahl von Studienteilnehmern war dieser beobachtete Unterschied jedoch nicht statistisch signifikant (**Veröffentlichung II**). Weitere und größere Studien zu diesem Aspekt könnten mehr Aufklärung bringen, zumal der Gebrauch antimikrobieller Mittel als einer der wichtigsten Risikofaktoren für die ESBL-EC-Kolonisierung in der Humanmedizin identifiziert wurde (Harris et al., 2007). Eine mögliche Strategie für eine weitere Studie könnte die Einbeziehung zusätzlicher Kliniken sein, um die Aussagekraft der Studie zu erhöhen (**Veröffentlichung II**). Allerdings konnte bereits gezeigt werden, dass die lokale MRE-Belastung tendenziell eher unterschiedlich zu sein scheint, insbesondere während eines Klinikaufenthalts (Apostolakos et al., 2017; Hordijk et al., 2020; Isgren et al., 2019; Shnaiderman-Torban et al., 2020). Dies könnte auch die Ursache für die lokalen Unterschiede in den Ergebnissen von bisher

veröffentlichten multizentrischen Studien sein (Marques et al., 2016; **Veröffentlichung II**). Darüber hinaus erschweren die Unterschiede in den technischen, strukturellen und hygienischen Gegebenheiten zwischen den veterinären Einrichtungen eine allgemeine Vergleichbarkeit der Standards innerhalb einer solchen Studie (**Veröffentlichung II**).

2.2.2. ESBL-EC-Phänotypen in hospitalisierten Pferden

Die vorherrschenden ESBL-Gene in den isolierten ESBL-EC dieser Studie waren *bla*$_{\text{CTX-M-1}}$ (59,7 %) und *bla*$_{\text{CTX-M-15}}$ (15,8 %) (**Veröffentlichung II**). Diese wurden bereits früher als dominierend in Proben von Pferden beschrieben (Hordijk et al., 2020; Isgren et al., 2019; Lupo et al., 2018; Walther et al., 2018). Darüber hinaus zeigten viele der in dieser Arbeit untersuchten Isolate nicht nur eine erweiterte β-Lactam-Resistenz, sondern auch eine verminderte Empfindlichkeit gegenüber anderen antimikrobiellen Substanzen. Die meisten der ESBL-EC-Isolate (51/85; 60%) erfüllten außerdem die Kriterien für MRE (Schwarz et al., 2010; **Veröffentlichung II**). Nicht selten sind MRE-ESBL-EC eine therapeutische Herausforderung für die Veterinärmedizin, da das Resistenzgen für ESBL-EC oft auf demselben Plasmid wie eine Resistenz gegen Aminoglykoside, Tetracycline und Trimethoprim/ Sulfamethoxazol liegt (Li et al., 2019).

Insgesamt konnten 21 verschiedene Sequenztypen (ST) für die im Studienzeitraum isolierten ESBL-EC identifiziert werden (**Veröffentlichung II**). Eine große Heterogenität der phylogenetischen Hintergründe für ESBL-EC bei Pferden wurde auch bereits in früheren Studien berichtet (Apostolakos et al., 2017; Schoster et al., 2020; Walther et al., 2018). In den hier untersuchten Proben konnten zum Teil verschiedene ESBL-EC-STen innerhalb einer einzigen Probe nachgewiesen werden (**Veröffentlichung II**). Die Einbeziehung verschiedener Phänotypen in das ESBL-EC-Screening von diagnostischen Proben sollte daher obligatorisch sein (Apostolakos et al., 2017). Die vorherrschenden phylogenetischen Linien in den ESBL-EC waren ST10, Sequenztypkomplex (STC) 86 (dazu gehören ST86, ST641 und ST453) und ST410. Weder ST86 noch ST641 wurden zuvor mit ESCL-EC aus klinischen Proben von Pferden in Verbindung gebracht (**Veröffentlichung II**). MRE-ESBL-EC der phylogenetischen Linie ST10 wurden bereits in Proben aus Fleisch, Geflügel, Wildtieren sowie in humanen klinischen Proben aus Spanien gemeldet (Díaz-Jiménez et al., 2020). Dies unterstreicht die Anpassungsfähigkeit dieser Bakterien sowie die Fähigkeit die Grenzen verschiedener Nischen und Wirte zu überschreiten. Auch EC der phylogenetischen Linie ST410 wurde bereits in Proben von verschiedenen Wirten nachgewiesen, darunter befinden sich Haus-, Nutz-, und Wildtiere, aber auch der Mensch (Falgenhauer et al., 2016; Fischer et al., 2017; Reynolds et al., 2019). Dem ST410 werden darüber hinaus hoch mobile und multiresistente Klone zugeschrieben, welche bereits in der Veterinär- als auch in der

Humanmedizin beschrieben wurden (Roer et al., 2018; Schaufler et al., 2016). Phylogenie und Habitat scheinen dabei die genetische Diversifizierung von *E. coli* zu beeinflussen. Der Erwerb von Genen und mobilen Elementen könnte dabei nicht nur über einen Austausch mit anderen Darmbakterien, sondern auch mittels gut etablierter Umweltbakterien geschehen (Touchon et al., 2020; **Veröffentlichung II**). Diese komplexen Anpassungsmechanismen fordern gezielte Strategien und Leitlinien, um die klinischen Herausforderungen in der antimikrobiellen Therapie von Pferden zu bewältigen und vor allem um HAI zu verhindern und die potenzielle Übertragung zwischen verschiedenen Quellen und Wirten, einschließlich Menschen, zu reduzieren (Apostolakos et al., 2017; Hordijk et al., 2020; Royden et al., 2019; Walther et al., 2014; **Veröffentlichung II**).

2.2.3. ESBL-EC-Übertragungsereignisse in hospitalisierten Pferden und lokale Dynamiken

Die Analyse der Phylogenie der identifizierten ESBL-EC zeigte eine enge Verwandtschaftsbeziehung zwischen vielen Isolaten. Diese Beobachtungen legen eine gemeinsame Quelle bzw. eine direkte oder indirekte Übertragung dieser ESBL-EC nahe (**Veröffentlichung II**). In den Nüsternabstrichen der hier untersuchten Tiere konnte u.a. ein einzelner Klon von zwei verschiedenen Pferden identifiziert werden, ein Hinweis, der die Annahme der lokalen Verbreitung verstärkt. Während beim Nachweis von ESBL-EC in Nüstern bei der Klinikaufnahme von einer vorherigen Kontamination ausgegangen werden kann, z.B. im Zusammenhang mit einer nasogastrischen Intubation (Walther et al., 2018), deuten die erhobenen Daten an t_1 und t_2 auf eine direkte oder indirekte Übertragung während des Klinikaufenthalts hin (**Veröffentlichung II**). Dies unterstützt auch die Ergebnisse früherer Untersuchungen, welche Tierkliniken als "Hotspots" für die Übertragung von MRE Enterobacterales identifizierten (Apostolakos et al., 2017; Walther et al., 2018; Wright et al., 2005). Eine Studie aus dem Jahr 2019 zeigte darüber hinaus, das die in EC identifizierten Resistenzen einer Gruppe nicht-hospitalisierter Pferde auch in EC aus Proben eines naheliegenden Viehbetriebes nachgewiesen werden konnten, was auch auf eine mögliche indirekte Übertragung hinweisen könnte (Kaspar et al., 2019). Die Aufklärung der Verbreitungs- und Übertragungswege von ESBL-EC in Pferdekliniken sollte daher ein wichtiger Bestandteil weiterführender Studien sein. Im Zeitraum von Januar bis August 2019 konnten eng verwandte Isolate der ST10 und STC86 sogar über mehrere Monate innerhalb der Klinik und über verschiedene Pferde nachverfolgt werden (**Veröffentlichung II**). Die lokale Akkumulation von MRE sowie die Anwesenheit von empfänglichen Patienten, welche durch die Gabe von Antibiotika oder anderen selektiven Mitteln

sensibilisiert sind, stellen vermutlich eine Hauptursache dieser Beobachtungen dar (**Veröffentlichung II**). Um die aktuellen Herausforderungen durch MRE und zoonotische Erreger in Pferdekliniken zu meistern, sollte das Bewusstsein für die Bedeutung der Hygiene sowie die Auswirkungen von antibakteriellen Therapeutika weiterhin geschärft werden.

2.3. Veröffentlichung III

Kauter A, Brombach J, Lübke-Becker A, Kannapin D, Bang C, Franzenburg S, Stoeckle SD, Mellmann A, Effelsberg N, Köck R, Guenther S, Wieler LH, Gehlen H, Semmler T, Wolf SA, Walther B. Antibiotic prophylaxis and hospitalization of horses subjected to median laparotomy: gut microbiota trajectories and abundance increase of Escherichia. Submitted to Frontiers in Microbiology, doi: 10.1101/2023.05.24.542119

Im Vergleich zu anderen domestizierten Tieren leiden Pferde besonders häufig an Erkrankungen des GIT, welche langfristig oft zum Tod führen. Im Allgemeinen wird die Zusammensetzung der Bakteriengemeinschaft im GIT als vorteilhaft und als Voraussetzung für die Gesundheit und das Wohlbefinden der Pferde angenommen (**Veröffentlichung I**). Vorangegangene Studien zeigten Hinweise darauf, dass die physiologisch-endogenen Mikrobiota im GIT der Pferde vor direkten oder indirekten Pathogen-induzierten Schäden schützen und dass diese Schutzwirkung bei verschiedenen enteralen Erkrankungen gestört sein könnte (Costa et al., 2012; Weese et al., 2015). Die Verabreichung von Antibiotika wie Penicillin (Baverud et al., 2003), Enrofloxacin oder Ceftiofur (Liepman et al., 2022) sowie Doxycyclin (Davis et al., 2006) kann die enterale mikrobielle Gemeinschaft stören und zu einem dysbiotischen Zustand führen (Costa et al., 2015). Obwohl die mediane Laparotomie bei akuten Fällen von Kolik ein häufig durchgeführter chirurgischer Eingriff bei Pferden ist, ist das Wissen über die Auswirkungen einer P/G PAP auf das Darmmikrobiom begrenzt. Klinische Studien zum Einfluss von Antibiotika auf das Darmmikrobiom werden aktuell hauptsächlich durch Querschnittsstudien abgebildet, während interventionelle oder longitudinale Ansätze und Vergleiche mit Kontrollgruppen oft fehlen (Zimmermann et al., 2021). Ein Vergleich zwischen krankheits- und arzneimittelbedingten Effekten ist daher schwierig (Zimmermann et al., 2021) und muss auch im Hinblick auf die Diskussion über mutmaßliche arzneimittelbedingte Effekte in der vorliegenden Arbeit klar und deutlich als Einschränkung adressiert werden (**Veröffentlichung III**). Da es sich in dieser Arbeit um ein reales Szenario und keinen Tierversuch handelt, kann der alleinige Effekt der P/G-Antibiose auf die Darmmikrobiota nicht vollkommen geklärt werden. Dennoch hat eine Studie aus der Humanmedizin gezeigt, dass die parentale Verabreichung von P/G eindeutig einen Einfluss auf das sich entwickelnde Darmmikrobiom von Säuglingen einschließlich der Shannon-Diversität und die Gesamtzusammensetzung hat (Reyman et al., 2022), so dass auch hier ein solcher Effekt zu erwarten ist (**Veröffentlichung III)**. Um diese möglichen Effekte genauer beschreiben zu können, wurden die Kotproben der Pferde aus der SSG (n=16) und der 5DG (n=15) sowie die Kotproben aus einer nicht-hospitalisierten Kontrollgruppe (n=10) im Hinblick auf Veränderungen in der Zusammensetzung der Bakterien (Mikrobiom) untersucht.

2.3.1. Diversität des fäkalen Mikrobioms hospitalisierter Pferde mit diagnostiziertem Kolik-Syndrom-Komplex unter dem Einfluss verschieden andauernder PAPs

Die zeitliche Veränderung in der Zusammensetzung der Darmmikrobiota sowie das Ausmaß von Störungen, welche durch die Gabe von Antibiotika möglicherweise verursacht wurden, sind die wesentlichen Aspekte dieses Studienabschnittes gewesen. Wie bereits in **Veröffentlichung I** beschrieben, ist die Zusammensetzung des enteralen Mikrobioms durch eine hohe Individualität und eine Vielzahl von externen Einflüssen geprägt. Dies gilt nicht nur für den Menschen, sondern auch für das Pferd (**Veröffentlichung I**).

Trotz dieser Einflüsse konnte für die in diesem Studienabschnitt untersuchten hospitalisierten Pferde bereits bei der Ankunft in der Klinik (vor jeglicher Antibiotikabehandlung (t_0)) eine im Mittel verminderte bakterielle Biodiversität festgestellt werden, was sich u.a. in einer verminderten Alpha-Diversität (Shannon-Diversitäts-Index (SDI)) im Vergleich zur Kontrollgruppe zeigte (**Veröffentlichung III**). Ähnliche Ergebnisse konnte eine Studie zeigen, welche geringere SDIs im Darmmikrobiom bei gastrointestinal erkrankten Pferden im Vergleich zu einer nicht betroffenen Kontrollgruppe feststellte (Park et al., 2021). Das fäkale Mikrobiom der in **Veröffentlichung III** untersuchten Proben der hospitalisierten Tiere zeigte die stärkste Veränderung, gekennzeichnet durch eine signifikante Abnahme des mittleren SDI, an t_1. Dieser Zeitpunkt liegt drei Tage nach erfolgter Laparotomie der hospitalisierten Tiere und markiert für die SSG den zweiten Tag nach der letzten Antibiotikagabe, während für die Teilnehmer der 5DG die Administration von P/G PAP von diesem Tag aus noch für zwei weitere Tage fortgesetzt wurde. An t_2 (10. Tag post-operativ) zeigten die wieder angestiegenen mittleren SDIs in beiden Studiengruppen deutliche Anzeichen für das Einsetzen einer Erholung im Mikrobiom, wobei die meisten der Studienteilnehmer den Ausgangszustand bereits wieder erreicht zu haben schienen, da der Unterschied zwischen t_0 und t_2 keine statistischen Unterschiede im Hinblick auf die mittlere Alpha-Diversität aufwies (**Veröffentlichung III**). Bemerkenswerterweise fand eine Studie an keimfreien Mäusen Hinweise darauf, dass die Erholung des Darmmikrobioms nach der Behandlung mit Antibiotika stark von der Ernährung, dem Gemeinschaftskontext und Umweltreservoiren abhängig zu sein scheint (Ng et al., 2019). Die Autoren stellten auch fest, dass die Verringerung von Bakterien in Umweltreservoiren den Prozess der Mikrobiomerholung beeinträchtigte (Ng et al., 2019). Diese Tatsache unterstreicht nicht nur die allgemeine Bedeutung der vorherrschenden Umweltbakterien in der unmittelbaren Umgebung von hospitalisierten Pferden, sondern verdeutlicht auch die Anfälligkeit der Darmmikrobiota von Pferden für die räumlich-zeitliche lokale Ausbreitung von Krankenhaus-assoziierten Krankheitserregern, einschließlich ESBL-EC, was

zu hohen Kolonisierungsraten führt, wie zuvor schon berichtet wurde (**Veröffentlichung II, Veröffentlichung III**).

Die Beta-Diversität bzw. Ähnlichkeit zwischen den einzelnen Proben wurde mittels der „Bray-Curtis distances“ auf Grundlage der identifizierten OTUs innerhalb der Proben ermittelt (**Veröffentlichung III**). Um die große und komplexe Menge dieser Daten für eine Analyse und Interpretation aufzubereiten, wurde anschließend eine (engl.) Principal Component Analysis (PCA) durchgeführt. Hierbei handelt es sich um eine Methode, welche die Vielzahl an statistischen Variablen in einem Datensatz durch wenige angenäherte aber aussagekräftige sogenannte Hauptkomponenten ersetzt und so starke Variationen und Muster visuell besser erkennen lässt (Lever et al., 2017).

Die beträchtlichen interindividuellen Abstände zwischen den Mikrobiomprofilen der Proben, welche aus der PCA der 5DG Pferde hervorgehen, sind zum Zeitpunkt t_1 erheblich kleiner als bei t_0, was auf eine ähnliche und konsistente Wirkung der Langzeit-PAP auf die Zusammensetzung der Darmmikroorganismen hinweist, welche spezifisch für die Langzeit P/G PAP ist (**Veröffentlichung III**). Diese Beobachtung deckt sich mit den Ergebnissen einer aktuellen Studie, in der die Auswirkungen häufig verwendeter Antibiotika auf das Darmmikrobiom gesunder menschlicher Probanden vor und nach der Behandlung untersucht wurde (Anthony et al., 2022). Die Autoren zeigten dabei mittels PCA medikamentenspezifische Profile in der Mikrobiom-Zusammensetzung über die Zeit (Anthony et al., 2022). In striktem Gegensatz zu den Ergebnissen der PCA der 5DG in der vorliegenden Studie zeigten die Datenpunkte der SSG vergleichsweise größere interindividuelle Unterschiede an t_1, was auf das Fehlen eines gemeinsamen selektiven Faktors hindeuten könnte (**Veröffentlichung III**). Diese Beobachtung könnte auch andere differenzierende Faktoren widerspiegeln, welche mit dem unterschiedlichen Individuum assoziiert sind, wie bspw. Appetit, Stress, Schmerz, Umweltbakterien, das Einsetzen der GIT-Funktion sowie die mikrobielle Wiederbesiedlung des GIT (diskutiert in **Veröffentlichung I**, **Veröffentlichung III**). Hier sind weitere gezielte Studien gefragt, um den Einfluss individueller sowie therapeutischer Faktoren auf das Mikrobiom der Tiere besser verstehen zu können.

2.3.2. Veränderungen des fäkalen Mikrobioms des Pferdes unter dem Einfluss verschiedener PAP-Regime

Um signifikante Veränderungen in der Zusammensetzung des Mikrobioms unter dem Einfluss der unterschiedlichen PAP aufzudecken, wurde eine Auswertung der Häufigkeiten einzelner Bakterienfamilien über die verschiedenen Probenziehungstage vorgenommen. In Verbindung mit den beiden unterschiedlichen PAP-Regimen war ein Anstieg in der Häufigkeit insbesondere für die

bakteriellen Familien Bacteroidaceae, Enterobacteriaceae und Pseudomonadaceae zu sehen (**Veröffentlichung III**). Veränderungen bei Bacteroidaceae sind im Zusammenhang mit Koliken beim Pferd bisher kaum beschrieben, jedoch gibt es Hinweise auf einen Zusammenhang zwischen *Bacteroides*, einer Gattung der Bacteroidaceae, und Koliken beim Pferd (Venable et al., 2013; **Veröffentlichung III**). Die Studie analysierte Kotproben von Vollblutpferden, die während der Kolik sowie 30 und 90 Tage später gesammelt wurden. Die Autoren berichteten über eine größere Häufigkeit von *Bacteroides spp*. in allen Proben, die während der Kolik gesammelt wurden, im Vergleich zu denen die 30 und 90 Tage später genommen wurden (Venable et al., 2013). Eine weitere Studie untersuchte Veränderungen im fäkalen Mikrobiom von Kolikern während eines Klinikaufenthalts, hier wurde ebenfalls ein erhöhtes Vorkommen von *Bacteroides* unter den erkrankten Tieren festgestellt (Stewart et al., 2021).

Multiresistente Enterobacterales sind in Pferdekliniken (Apostolakos et al., 2017; Shnaiderman-Torban et al., 2020; Walther et al., 2018) bzw. im Zusammenhang mit chirurgischen Wundinfektionen ((engl.) surgical site infections, kurz: SSI) nach einer Laparotomie bei Pferden (Dziubinski et al., 2020; Isgren et al., 2017) bereits häufig beschrieben worden. Die vor allem am Probentag t_1 festgestellte erhöhte Häufigkeit von Enterobacterales im Kot der hospitalisierten Tiere könnte ein möglicher Grund für das erhöhte Risiko der Entwicklung von SSI durch resistente Infektionserreger für die Pferdepatienten sein (**Veröffentlichung III**). Auch im Zusammenhang mit Laminitis wurde bereits von einer relativ erhöhten enteralen Häufigkeit von Enterobacterales bei Pferden berichtet (Milinovich et al., 2008).

Ein weiterer signifikanter Anstieg konnte in der Familie der Pseudomonadaceae von t_0 nach t_1 festgestellt werden (**Veröffentlichung III**). Eine Zunahme der relativen Häufigkeit dieser Familie von Mikroorganismen steht möglicherweise in Verbindung mit verschiedenen gastrointestinalen Erkrankungen und wurde bereits als Marker für Veränderungen des Darmmikrobioms beim Menschen beschrieben (Alam et al., 2020; Chamorro et al., 2021).

Um den direkten Einfluss der akuten Erkrankung und anderer Faktoren auf das GIT-Mikrobiom der Pferde besser untersuchen zu können, wurde die Häufigkeit verschiedener Bakterien in den Kotproben beider Studiengruppen im Vergleich zu einer Kontrollgruppe (t_0) ausgewertet (**Veröffentlichung III**). Dabei konnten deutliche Abweichungen für OTUs aus 15 verschiedenen Bakterienfamilien identifiziert werden. Insgesamt war ein gemeinsamer zeitlicher Verlauf für Bacteroidaceae, Enterobacteriaceae und Pseudomonadota erkennbar, mit einem auffallend erhöhten Anteil in fast allen Proben zu t_1 und abnehmenden, aber immer noch über dem Basiswert liegenden, OTU-Zahlen zu t_2. Darüber hinaus konnte eine geringere Häufigkeit von Lachnospiraceae bei Pferden mit diagnostizierter Kolik im Vergleich zu den Pferden der Kontrollgruppe beobachtet werden (**Veröffentlichung III**). Dies stimmt auch mit den von Steward

et al. 2019 berichteten Ergebnissen für an Kolik-leidenden Pferden überein (Stewart et al., 2019). Außerdem wurde ein verringerter Anteil an Lachnospiraceae im fäkalen Mikrobiom auch bei Pferden mit enteralem Erkrankungsbild beschrieben (Costa et al., 2012; Weese et al., 2015).

2.3.3. Veränderungen des Mikrobioms im Zusammenhang mit erhöhten ESBL-EC-Kolonisierungsraten

Die allgemeinen Auswirkungen der P/G PAP auf das Darmmikrobiom der Pferde fielen ausgeprägter aus als zunächst im Zuge einer parenteralen Verabreichung erwartet wurde (Stöckle et al., 2021; Zhang et al., 2013; **Veröffentlichung III**). Der gemeinsame Einfluss von Kolik, Klinikaufenthalt, Laparotomie sowie Verabreichung einer PAP führte zu einem Anstieg in den relativen Häufigkeiten der Gattungen *Escherichia* und *Bacteroides* (**Veröffentlichung III**).

Die meisten der untersuchten Pferde wiesen bei der Aufnahme in die Klinik eine geringe relative Häufigkeit von *Escherichia* im fäkalen Mikrobiom auf, welche an t_1 deutlich anstieg. Diese Beobachtung steht im Einklang mit dem Verlauf des Anstiegs der ESBL-EC Besiedlungsraten beider Studiengruppen über die Zeit (**Veröffentlichung II**). Dies könnte auch auf eine lokale Ausbreitung von ESBL-EC während der P/G PAP hinweisen, welche wohlmöglich durch einen Selektionsvorteil im GIT der Pferde beeinflusst wird, wodurch sich einzelne ESBL-EC-Linien bevorzugt vermehren und verbreiten können. Dies führt nicht nur zu steigenden Kolonisierungsraten (**Veröffentlichung II**), sondern auch zu einer unvermeidbaren Umweltkontamination über z.B. durch den Kot kontaminierte Einstreu in den Unterbringungen der Pferde (**Veröffentlichung III**). Da die unmittelbare Umgebung zu den Hauptquellen von GIT-assoziierten Bakterien während einer Wiederbesiedlung des Mikrobioms gehört (Ng et al., 2019), scheinen vor allem Umweltquellen eine wichtige Rolle in Bezug auf die ESBL-EC Kolonisierungsraten zu spielen.

Nach einer Behandlung mit β-Laktamen konnte im GIT-Mikrobiom beim Menschen nicht nur eine Zunahme von Enterobacteriaceae, sondern auch eine Zunahme von Bacteroidaceae festgestellt werden, wie eine Übersichtsarbeit zeigte (Patangia et al., 2022), eine Beobachtung die ebenfalls mit den im Rahmen dieser Arbeit publizierten Ergebnissen übereinstimmt (**Veröffentlichung III**). Da für *Bacteroides spp*. eine intrinsische Resistenz (CLSI, 2020; Pumbwe et al., 2006) beschrieben wird, kann zumindest die Auswirkung eines behandlungsbedingten Selektionsvorteils in Betracht auf den relativen Anstieg der Abundanz in den meisten Kotproben der hospitalisierten Tiere an t_1 angenommen werden (**Veröffentlichung III**). Obwohl viele *Bacteroides*-Arten eine entscheidende Rolle beim Abbau von Polysacchariden in einer pflanzlichen Ernährung spielen (Cheng et al., 2022; Pereira et al., 2021), wurde die spezifische Bedeutung und Rolle der verschiedenen Arten bei der Fermentation im Darm noch nicht untersucht. Weitere Forschung zu diesem Thema, einschließlich

der Analyse metabolomischer Profile aus Metagenom-Sequenzierungsprojekten, ist erforderlich, um die Zusammenhänge besser zu verstehen (**Veröffentlichung III**).

3. Ausblick

In dieser Arbeit sollte eine einmalige perioperative P/G Antibiose im Zuge eines operativen Eingriffs nach diagnostizierter Kolik der bislang üblichen fünf-Tage-Antibiose gegenübergestellt werden und dabei der Einfluss auf die Häufigkeit von ESBL-EC und die Veränderungen im enteralen Mikrobiom der Pferde untersucht werden.

In Abbildung *4* sind die in dieser Studie beobachteten Einflüsse und Effekte der Kolikoperation, Hospitalisierung und in diesem Zusammenhang verabreichten PAP auf das Darmmikrobiom der Pferde grafisch zusammengefasst:

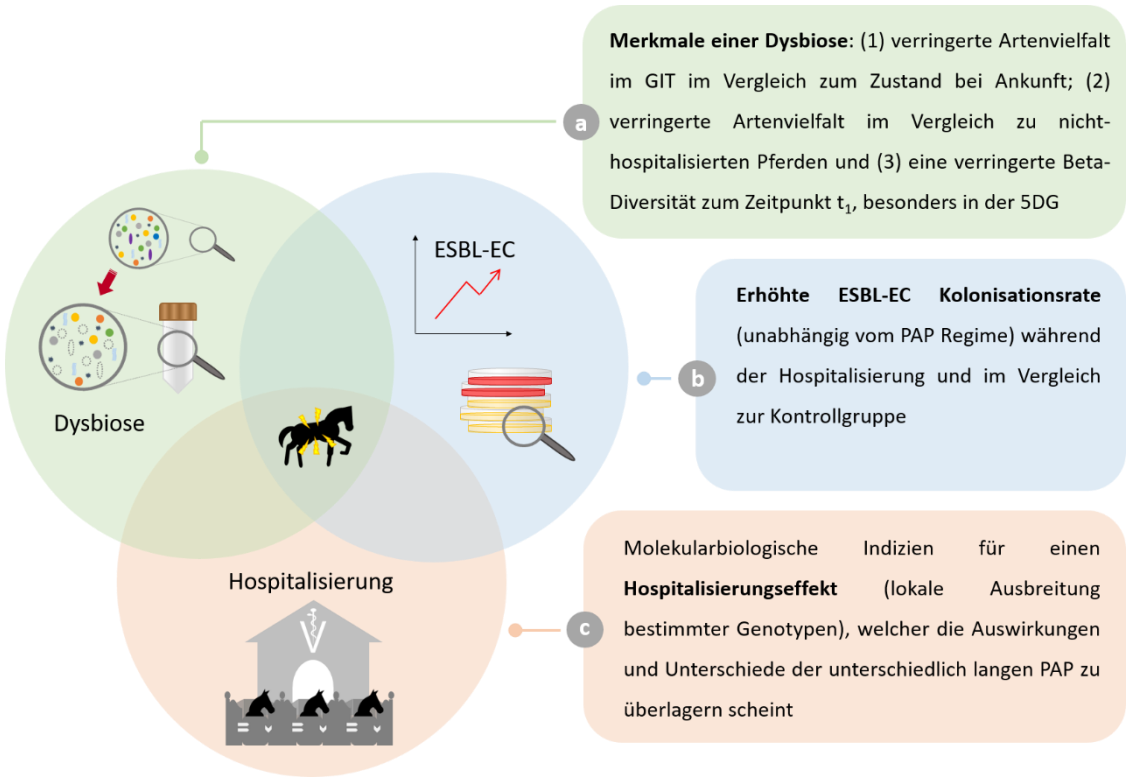

Abbildung 4| **Grafische Darstellung der verschiedenen Einflüsse von Kolikoperation, Hospitalisierung und PAP auf das Darmmikrobiom des Pferdes.** Neben den Merkmalen einer Dysbiose (a) konnte ebenfalls eine erhöhte Rate an ESBL-EC (b) in den Proben der hospitalisierten Pferde im Vergleich zur nicht-hospitalisierten Studiengruppe festgestellt werden. Die molekularbiologischen Untersuchungen ergaben Hinweise auf nahe verwandte ESBL-EC Isolate, welche über einen längeren Zeitraum und in verschiedenen Tieren nachgewiesen werden konnten. Dieser Hospitalisierungseffekt (c) führte vermutlich auch zu einer Überlagerung des Einflusses der unterschiedlich lang verabreichten PAP-Regime.

Der durch antimikrobielle Therapeutika induzierte Diversitätsverlust im GIT-Mikrobiom ist nicht selten von Durchfallerkrankungen oder Antibiotika-assoziierten Infektionen begleitet (Barr et al., 2013; Ramirez et al., 2020). Einige Studien richteten in der Vergangenheit ihre Aufmerksamkeit deshalb auf den Versuch, fäkale Mikrobiota beim Pferd zu transplantieren, um mögliche Ungleichgewichte im GIT der Tiere aufgrund von bspw. Antibiotikabehandlungen zu vermindern (Costa et al., 2021; Mullen et al., 2018). Eine erst kürzlich erschienene Studie bewertete dabei die

klinischen und mikrobiologischen Auswirkungen einer fäkalen Mikrobiom-Transplantation bei Pferden mit Diarrhöe (McKinney et al., 2021). Die Autoren konnten dabei eine Abmilderung des Schweregrades der Diarrhöe für Tiere, welche an drei aufeinanderfolgenden Tagen behandelt wurden, im Vergleich zu einer unbehandelten Kontrollgruppe zeigen (McKinney et al., 2021). Anders fiel eine Studie zur täglichen fäkalen Mikrobiomtransplantation beim Pferd aus, welche eine Metronidazol-induzierte Dysbiose zu verhindern versuchte (Kinoshita et al., 2022). Hier reichte die zusätzliche einmalige tägliche Gabe einer fäkalen Mikrobiotatransplantatsuspension nicht aus, um die Metronidazol-induzierten Veränderungen im Mikrobiom signifikant zu vermindern. Dabei blieb die Frage offen, ob die unzureichende prophylaktische Wirkung des Mikrobiomtransplantats in dieser Studie im Zusammenhang mit einer zu geringen Menge, Häufigkeit oder einem unpassendem Verabreichungszeitpunkt stand (Kinoshita et al., 2022). Die ausführliche Evaluierung und Anpassung von fäkalen Transplantationsprotokollen anhand der neuesten Erkenntnisse in der Mikrobiomforschung könnten dahingehend weitere Fortschritte bringen, die mögliche Dysbiose im Mikrobiom der Pferde nach einer Antibiotikabehandlung in Zukunft möglicherweise besser therapieren zu können.

Einen anderen Ansatz lieferte eine erst 2021 erschienene Studie zum Effekt von bakteriostatischen und bakterioziden Wirkstoffen auf die mikrobielle Gemeinschaft im GIT (Maier et al., 2021). Die Autoren stellten fest, dass eine strikte und allgemeine Eingruppierung der Wirkung von Antibiotika in bakteriostatisch und bakteriozid kaum durchzuführen ist, da diese Effekte sehr spezifisch für einzelne Spezies zu sein schienen. Außerdem bemerkten sie eine schnellere Erholung des Mikrobioms beim Einsatz von lediglich bakteriostatisch wirkenden Therapeutika (Maier et al., 2021). Am bemerkenswertesten waren jedoch die Untersuchungen von spezifischen Antagonisten zum Schutz einzelner Bakterien vor einer bakterioziden Wirkung eines Antibiotikums. So konnte bspw. die Gabe des Antikoagulants Dicumarol und des Urikusurikums Benzbromaron Bakterien der Spezie *Bacteroides vulgatus* vor der Wirkung des Erythromycins bewahren. Dieses Konzept könnte langfristig einen Weg aufzeigen, wie zukünftig die spezifischen Kollateralschäden von Antibiotika auf einzelne Kommensalen möglicherweise abzumildern sind (Maier et al., 2021).

Die Evaluierung der Verbreitung von ESBL-positiven Enterobacterales, auch innerhalb von Tierkliniken, ist bereits länger ein beständiger Schwerpunkt in vielen Studien (Royden et al., 2019; Thomson et al., 2022), auch um das Auftreten von nur schwer zu therapierenden Infektionen zu vermeiden. Molekulare epidemiologische Untersuchungen legen nahe, dass bei der Übertragung resistenter Bakterien auf den Menschen nicht zuletzt tierische Quellen eine wichtige Rolle zu spielen scheinen, wie eine Studie zur Herkunftsbestimmung von ESBL-EC Isolaten innerhalb Deutschlands zeigte (Perestrelo et al., 2022). Alle untersuchten tierischen Quellen dieser Studie (Hühner, Rinder, Schweine, Hunde und Pferde) teilten dabei die meisten Subtypen ESBL-positiver

Bakterien mit der Allgemeinbevölkerung (Perestrelo et al., 2022). Dahingegen konnte der berufsbezogene Kontakt mit bestimmten Tierarten (u.a. Hunde, Katzen, Pferde) nicht mit einer signifikanten ESBL-EC oder *Klebsiella pneumoniae* (*K. pneumoniae*) -Übertragung assoziiert werden, wie eine Studie zu ESBL/pAmpC positiven EC und *K. pneumoniae* bei Mitarbeitern im Veterinärwesen in den Niederlanden zeigte (Meijs et al., 2021). Darüber hinaus ergab die Charakterisierung und Untersuchung von Risikofaktoren für eine ESBL-Enterobacterales Kolonisierung in einer Pferdeklinik im Zusammenhang mit einem Ausbruch von *K. pneumoniae*-Infektionen, dass neben der Dauer des Klinikaufenthaltes u.a. auch der Gebrauch von nasogastralen Sonden als Risikofaktor im Zusammenhang mit ESBL-Enterobacterales-positiven Pferden stand (Thomson et al., 2022). Ein rascher Anstieg in den ESBL-Enterobacterales-Kolonisierungsraten im Zuge einer Hospitalisierung und Verabreichung von Antiinfektiva wurde nicht nur, wie bereits schon hier berichtet, beim Pferd beobachtet, sondern auch erst kürzlich beim Menschen deutlich beschrieben (Lewis et al., 2022). Eine Studie untersuchte dabei die Besiedlungsdynamiken von ESBL-Enterobacterales im Darm von Erwachsenen in Malawi. Die Autoren stellten bei einer Gegenüberstellung verschiedener Faktoren fest, dass der Einfluss der Antibiotika auf den Anstieg in den ESBL-Enterobacterales-Kolonisierungsraten größer zu sein scheint, als der Einfluss der Hospitalisierung an sich (Lewis et al., 2022). Die Autoren gehen weiterhin davon aus, dass aufgrund der anhaltenden Wirkung von verabreichten Antibiotika kurze Behandlungen eine ähnliche Wirkung wie längere Behandlungen in Bezug auf die ESBL-Enterobacterales-Übertragung zeigen könnten (Lewis et al., 2022). Dies könnte sich auch in den im Rahmen dieser Arbeit berichteten in der Regel nur sehr geringen Unterschieden zwischen den zwei untersuchten PAP-Regimen widerspiegeln (**Veröffentlichung II, III**). Die Ausbreitung von ESBL-Enterobacterales scheint dabei vor allem durch den selektiven Vorteil der Bakterien gegenüber den häufig verwendeten β-Laktam-Antibiotika gefördert zu werden (**Veröffentlichung II, III**). Eine andere Studie beschäftigte sich weiter mit den Kurz- und Langzeiteffekten der Hospitalisierung und oralen Gabe von Sulfadiazin/ Trimethoprim auf das Mikrobiom in Kotproben vom Pferd (Theelen et al., 2023). Laut den Autoren der Studie kam es während der Hospitalisierung ohne den Einfluss einer PAP über acht Tage weder zur Beeinflussung der Zusammensetzung der Mikrobiota, noch zu einer Veränderung der Abundanz von Resistenzgenen innerhalb der Proben (Theelen et al., 2023). Erst unter dem Einfluss des Antibiotikums wurde von einer verminderten Alpha-Diversität in der Mikrobiota-Zusammensetzung der Proben sowie von einer erhöhten Abundanz an Resistenzgenen berichtet (Theelen et al., 2023). Die sehr geringe Studienpopulation (6 Tiere) und die Tatsache, dass es sich um klinisch gesunde Tiere handelte, muss jedoch klar als Limitation in der Interpretation dieser Studienergebnisse benannt werden. Allerdings würden diese Ergebnisse auch die Robustheit eines gesunden Mikrobioms gegenüber äußeren Einflüssen unterstreichen,

ebenso wie den bereits beschriebenen immensen Einfluss durch Faktoren wie Nahrung und Kontakt mit Umweltreservoiren während der „Wiederbesiedlung“ des Mikrobioms nach einer antimikrobiellen Therapie (**Veröffentlichung III**).

Das derzeitige begrenzte Wissen zu den einzelnen Funktionen der Mikrobiota im equinen Mikrobiom sowie dessen Individualität sind wohl als eine der größten Schwierigkeiten zu adressieren. Sequenzier- und Hochdurchsatz-Methoden ermöglichen die zeitnahe Erfassung aller in einer Probe vorhandenen Gene (Metagenom) und könnten zukünftig in groß angelegten Studien weitreichende und tiefere Einblicke in die Beziehung zwischen Gesundheit und Mikrobiom, nicht nur beim Pferd, sondern auch im Hinblick auf ein One-Health-Konzept geben (Lemos et al., 2022). Die in dieser Studie gesammelten Ergebnisse und gewonnenen Erkenntnisse stellen jedoch einen ersten wichtigen Schritt in der Etablierung von Antibiotic Stewardship-Programmen in Pferdekliniken dar und könnten somit einen langfristigen Einfluss auf die lokale Verbreitung von MRE haben.

4. Veröffentlichungen

4.1. **Veröffentlichung I**

The gut microbiome of horses: current research on equine enteral microbiota and future perspectives

Anne Kauter, Lennard Epping, Torsten Semmler, Esther-Maria Antao, Dania Kannapin, Sabita D. Stoeckle, Heidrun Gehlen, Antina Lübke-Becker, Sebastian Günther, Lothar H. Wieler, Birgit Walther

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Animal Microbiome

REVIEW Open Access

The gut microbiome of horses: current research on equine enteral microbiota and future perspectives

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Abstract

Understanding the complex interactions of microbial communities including bacteria, archaea, parasites, viruses and fungi of the gastrointestinal tract (GIT) associated with states of either health or disease is still an expanding research field in both, human and veterinary medicine. GIT disorders and their consequences are among the most important diseases of domesticated Equidae, but current gaps of knowledge hinder adequate progress with respect to disease prevention and microbiome-based interventions. Current literature on enteral microbiomes mirrors a vast data and knowledge imbalance, with only few studies tackling archaea, viruses and eukaryotes compared with those addressing the bacterial components.

Until recently, culture-dependent methods were used for the identification and description of compositional changes of enteral microorganisms, limiting the outcome to cultivatable bacteria only. Today, next generation sequencing technologies provide access to the entirety of genes (microbiome) associated with the microorganisms of the equine GIT including the mass of uncultured microbiota, or "microbial dark matter".

This review illustrates methods commonly used for enteral microbiome analysis in horses and summarizes key findings reached for bacteria, viruses and fungi so far. Moreover, reasonable possibilities to combine different explorative techniques are described. As a future perspective, knowledge expansion concerning beneficial compositions of microorganisms within the equine GIT creates novel possibilities for early disorder diagnostics as well as innovative therapeutic approaches. In addition, analysis of shotgun metagenomic data enables tracking of certain microorganisms beyond species barriers: transmission events of bacteria including pathogens and opportunists harboring antibiotic resistance factors between different horses but also between humans and horses will reach new levels of depth concerning strain-level distinctions.

Keywords: Horse, Microbiome, Gastrointestinal tract, Microbiota, Disease, Health

Equine microbiota and microbiomes: what we know so far

Humans and animals have a unique set of diverse microorganisms, an individual fingerprint. The complex and multi-levelled interactions between these resident microorganisms with respect to disease risks, health preservation, immunity and therapeutic possibilities are currently expanding research fields in both, human– and veterinary medicine. The intestinal tract of Equidae contains a diverse community of microorganisms that consists of fungi, parasites, protozoa, archaea, viruses and bacteria [1]. This entirety of different microorganisms associated with a distinct space is known as the microbiota, while the corresponding entity of genetic material is referred to as microbiome [2]. While this particular distinct and individual composition of a broad range of microorganisms includes essential nutrition suppliers and immune response supporters [3], it also contains taxa capable of causing disease [4]. All Equidae belong to a family of herbivorous mammals that possess a certain hindgut (caecum and colon) microbiota, enabling forage utilization for optimal nutrition. These microbes provide

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a substantial proportion of the horses' daily energy needs through the fermentation of plant material to short chain fatty acids such as acetate, propionate, and butyrate [5, 6]. Consequently, gastrointestinal disturbance in the equine microbiota can result in alteration of fermentation patterns and, ultimately, metabolic disorders [7]. While knowledge about the role of archaea, viruses and eukaryotes residing within the GIT and their contribution to a healthy human microbiome is limited [8], even less data is available for horses, mirrored only by a few studies as shown in Table 1.

Within their enteral tract, horses are able to host up to 10^{15} bacterial cells [9] with the majority of bacteria residing in the colon, especially within the comparatively enlarged caecum [10]. The degradation of non-digestible cellulosic and hemi-cellulosic forage components by these microorganisms is crucial for the bioavailability of energy and other essential nutritional needs in horses [9].

Several diseases including cardiovascular disorders [11, 12], inflammatory bowel disease [13], diabetes [14–16], rheumatoid arthritis [17], depression [18] and progression of cancer [19–22] have, among others, been associated with distinct changes in human intestinal microbiomes in recent years. Compositional changes of the equine microbiota were similarly investigated with respect to its impact on certain diseases such as equine grass sickness [23], colitis and laminitis [24–26]. Moreover, the effects of distinct diets and dosage forms have been studied in elderly horses and horses in training [27, 28]. In the years that followed, maps of the equine microbiome [29–31] and the putative impact of probiotics such as *Lactobacilli* and *Bifidobacteria* were explored [32, 33]. Another recent focus of research is to unveil the putative composition of an equine hindgut "core" microbiota. This core microbiota should mirror the stable, consistent bacterial components including key microorganisms and their functions [30, 34–36]. In yet another study, the impact of antimicrobial treatment and anesthesia was investigated with respect to their role in shaping equine microbial composition [37, 38].

In this review we aim to provide an overview about the **i**) techniques used or available for equine microbiome exploration **ii**) current knowledge on equine hindgut microbiota with an emphasis on bacterial components **iii**) traits and factors which might influence equine microbiome diversity and composition and **iv**) future trends and perspectives in this field.

How to study microbial communities: techniques currently available to define the equine enteral microbiome

For interpretation of studies on the microbiome composition, including those of hindgut fermenters such as horses (Additional file 1), it is necessary to understand the different technologies currently used for data generation and exploration. Until recently, the identification of intestinal microorganisms was performed by culture-dependent methods limiting the outputs to cultivable species only [39]. These methods are, however, slowly being replaced and/or complemented by new comprehensive approaches such as "Culturomics", a method which includes multiple growth conditions to a subdivided original sample together with extended incubation times. In combination with rapid identification methods for bacteria such as Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF-MS), a fast and extended overview on cultivable bacterial components of a sample of interest is possible. Mass spectra of so far unidentified species could be generated and assigned by the additional use of 16S rRNA sequencing [40]. Consequently, Culturomics can be seen as a kind of "rebirth" of culture-based techniques in microbiology [41], producing results which are easy to combine with other methods commonly used to study animal microbiomes (Fig. 1).

Overall, high-throughput sequencing approaches are currently the most predominant techniques to investigate microbiomes, in clinical research as well as in environmental science [42, 43]. The recent developments in DNA sequencing technologies, also referred to as next-generation sequencing (NGS), now allow researchers to study complex biological samples based on sequence information on a large scale [44]. In general, DNA is first purified from the samples and DNA sequencing is then used to characterize the associated taxa, employing either a ubiquitous marker gene such as the 16S rRNA gene for bacteria, the 18S rRNA gene for eukaryotes or an internal transcribed spacer (ITS) DNA present between rRNA genes for fungi. Alternatively, all DNA in a given sample is sequenced by use of shotgun metagenomics sequencing [45]. Since NGS allows for cost-effectiveness, sufficient resolution and sequencing depth for many research questions, this is one of the most commonly used techniques in medical- (food)hygiene- and environmental metagenomics studies [39].

One method to explore microbial compositions is NGS of the bacterial ubiquitous ~ 1500 base pair 16S rRNA gene made up of nine hypervariable regions flanked by conserved sequences [46]. Here, primers are used to define resulting amplicons covering the hypervariable regions which then differ in amount and base composition per sample under investigation. Based on the nucleotide sequence similarity, these sequences are clustered into Operational Taxonomic Units (OTU) [47].

To ascribe taxonomic identities of a certain bacterial community, NGS results are compared to 16S rRNA gene sequence databases available, including Greengenes [48] and Silva [49]. With its conserved and variable sequence regions evolving at very different rates, the 16S rRNA sequences provide reliable data for investigating both close

Table 1 Microorganisms with nourishment-associated activity in the gastro enteral tract of horses

Kingdom	Family	Genus	Species	Putative effects	Ref.
Bacteria	*Ruminococcaceae*	*Ruminococcus*	spp.	cellulolytic, fibrolytic bacteria	[167, 168]
			favefaciens	plant wall degradation	[169]
			albus	plant wall degradation	[169, 170]
	Fibrobacteraceae	*Fibrobacter*	*succinogenes*	monosaccharide and glycoside degradation	[169–172]
			intestinalis	plant wall degradation	[171]
	Streptococcaceae	*Streptococcus*	spp.	amyloytic[a]	[173]
			bovis/equinus	L-lactate producer	[174]
	Lactobacillaceae	*Lactobacillus*	*salivarius/ mucosae*	L-lactate producer, decarboxylating amino acids, vascoactive amines	[174], [137]
			bulgaricus/ delbrueckii	L-lactate producer	[174]
			crispatus	lactic acid bacteria	[175]
			johnsonii	lactic acid bacteria	[175]
			reuteri	lactic acid bacteria	[175]
			equigenerosi	lactic acid bacteria	[176]
			hayakitensis	lactic acid bacteria	[176]
			buchneri	lactic acid bacteria	[176]
			vitulinus	lactic acid bacteria	[176]
	Acidaminococcaceae	*Mitsuokella*	*jalaludinii*	D-lactate producer	[174]
		Phascolarctobacterium	spp.	fibre fermenters[b]	[168]
	Veillonellaceae	*Veillonella*	*gazogenes/ alcalescens*	lactat utilizing bacteria	[177]
	Lachnospiraceae	*Butyrivibrio*	spp.	cellulolytic, fibrolytic[c]	[167]
			fibrosolvens	amylolytic	[173]
		Blautia	spp.	fibre fermenters	[168]
	Clostridiaceae	*Clostridium*	spp.	cellulolytic, fibrolytic[d]	[167]
	Eubacteriaceae	*Eubacterium*	spp.	cellulolytic, fibrolytic	[167]
	Prevotellaceae	*Prevotella*	spp.	fibre fermenters	[168]
	Succinivibrionaceae	*Ruminobacter*	*amylophilus*	amylolytic	[173]
	Enterococcaceae	*Enterococcus*	*faecalis*	amylolytic	[173]
Fungi				fiber degradation	[178]
	Neocallimastigaceae	*Piromyces*	*equi*	cellulose degradation	[179]
Protozoa				hemicellulose, pectin degradation	[99]
Bacterio-phages				regulating bacterial species distribution	[180]
Archaea				methanogens[f]	[80], [81]

and distant phylogenetic relationships, and allow a precise assessment of phylogenetic relatedness of organisms [50]. Currently, a broad panel of bioinformatic tools designed for sequencing data analysis are available, including many which are open source and easy to operate [47]. Commonly used software to analyze 16S rRNA data from food/environmental samples include QIIME (Quantitative Insights into Microbial Ecology) [51], mothur [52], and USEAR (ultra-fast sequence analysis) [53]. These tools assign the sequences to specific taxonomic levels based on clustering for OTUs at different sequence identity thresholds.

However, there still are clear limitations when using NGS 16S rRNA based identification of bacteria beyond the family level [54], since current sequencing read lengths with Illumina technology only cover a region of around

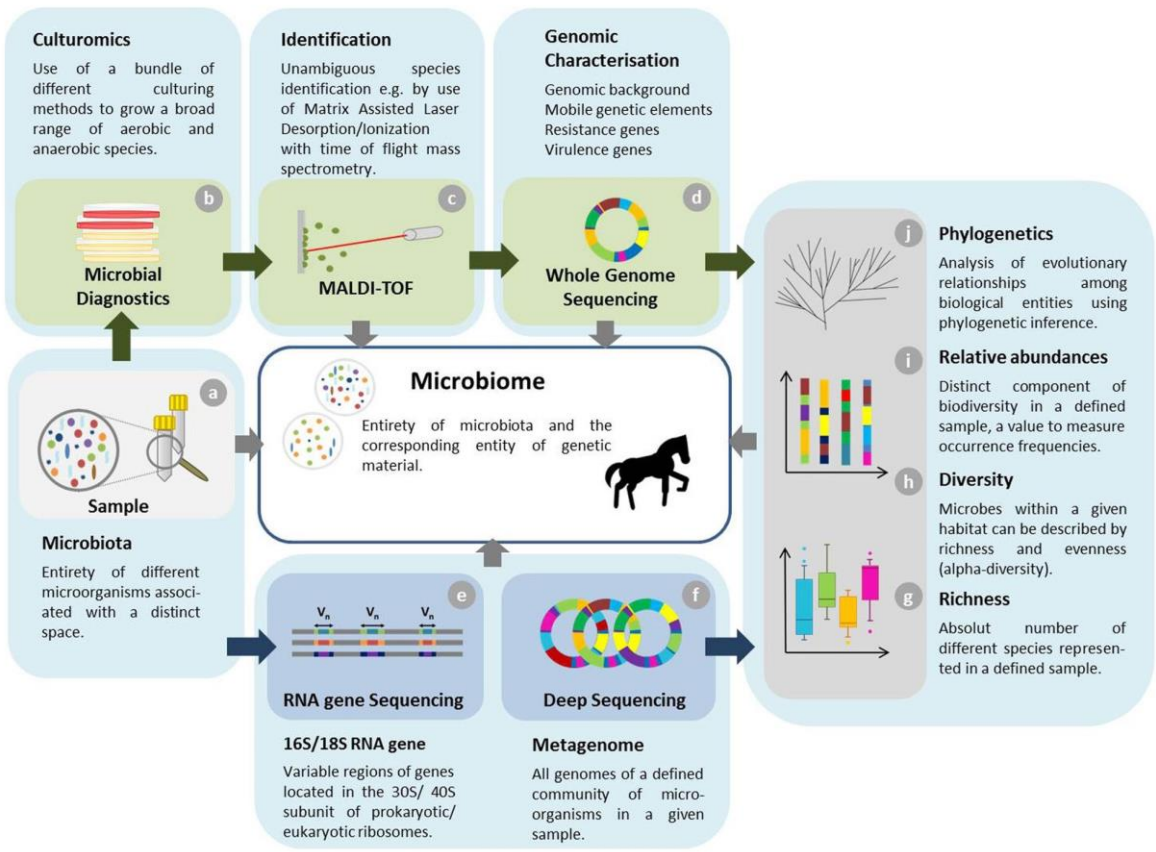

Fig. 1 Workflow and synergistic application of diffent techniques to study enteral microbiomes. Integrative and synergistic workflow to study equine microbiomes starting with dividing the fresh sample (**a**) for two general processing's, microbial diagnostic in terms of culturomics (**b**-**d**) [157] and DNA sequencing approaches (**e** and **f**) for population analysis (**g**-**i**). A broad range of different aerob and aerobic culture conditions are used to initiate growth for microbial diagnostic (**b**), followed by rapid species identification by MALDI-tof mass spectrometry (**c**). Genome sequencing (**d**) allows (novel) species identification in case MALDI-tof provided no confident result or if resistance- and virulence encoding genes [158, 159] or other factors are of particular interest within a species. Both information sources allow identification of bacterial species present in the horse microbiota and their growth conditions. The second part of the sample should be stored native at − 80 °C until DNA extraction starts for either sequencing of variable regions of 16S/18S rRNA gene (**e**) allowing characterizing and quantifying taxonomic entities or sequencing of all genomes (metagenome) present in a sample (**f**). Further bioinformatics include description of richness (**g**), diversity indices (**h**) [160–162], relative abundances (**i**) and phylogenetics (**j**). Combination of classical diagnostics on a large scale and different techniques available to generate genomic data enable deep insights into microbiome composition and characteristics [163]

460 bp mostly from the V3 and V4 region while a full-length or near full-length 16S rRNA sequence is needed for a confident taxonomic assignment of genus and species [50]. Since it is known that bacterial species differ with respect to their copy numbers of the 16S rRNA gene from one to 15 and more [55], amplification could lead to a bias considering semi quantitative proportions (relative abundances) in complex communities [56]. Moreover, the selection of primer sets used for amplification of the 16S rRNA gene might result in over- or underrepresentation of distinct bacterial species [57].

Shotgun sequencing of whole genome DNA samples provide the most complete information on the entire gene pool within a sample while the high amount of generated data requires substantial efforts of bioinformatics in sequence assembly, mapping and analyses [39]. In principle, the method is quite similar to those used for sequencing a single bacterial genome [58], but the output data consists of all genome sequences present in a given complex sample including archaea, bacteria, fungi and viruses. A recent study demonstrated that shotgun whole genome sequencing has multiple advantages compared with the 16S amplicon method such as enhanced detection of bacterial species, increased detection of diversity and abundance as well as increased prediction of genes relevant for example for antimicrobial resistance or virulence determination. In addition, providing sequence data of the whole genome of the present

microorganisms in combination with whole genome reference databases greatly improved the accuracy of species detection [59]. A comprehensive overview on current methods frequently used for microbiome surveys together with means for beneficial complementation of different techniques and analysis methods is provided in Fig. 1.

However, creating valid results from shotgun sequencing of complex microbiomes is still challenging and computationally intensive [60]. Till date, open databases available to assign genomic data by mapping metagenomics reads provide more primary whole genome sequencing (WGS) data for reference- and pathogenic strains, while colonizing or non-pathogenic bacteria had less often been sequenced in the past [61]. Consequently, a significant proportion of shotgun sequences is dedicated to "microbial dark matter" of gut microbiomes, since suitable reference genomes of non-cultivable and/or non-pathogenic bacteria are not available for assignments [44]. In addition, methodical standardization and the development of specific pipelines for data analysis and –reproducibility are still an ongoing matter of discussion [62]. Microbiome research reliability and -development depend on reliable data at free disposal. In fact, providing raw sequencing data lacking corresponding sets of metadata hinders any attempt to reproduce the original study results [63]. As a consequence, databases like NCBI SRA (https://www.ncbi.nlm.nih.gov/sra) were established for storing and sharing sequencing data. Taken together, NGS technology developments have shown great progress in recent years, but technical issues still exist, mainly related to the need of continuously updated databases, specific bioinformatic tools, and functional correlations [62].

In 2012, first studies addressing the equine microbiome were published, reporting on 2–6 horses providing up to 16 specimens subjected to microbiome analysis. Since then, the numbers of animals under investigation, samples and data processing, as well as evaluation opportunities have increased dramatically. Additional file 1 provides a comprehensive overview on microbiome surveys in horses published so far (2018).

Microbiomes' markers: species abundances, sample richness and diversities

One of the most important goals of many microbiome surveys is to explore and describe differences in the relative abundances of bacterial taxa induced by environmental changes [64]. As the abundances generated by NGS technology are semi-quantitative by definition, the observed dynamics may not accurately reflect those of the actual taxon densities, a fact that was shown by way of comparison of single-cell counting by use of flow cytometry with 16S rRNA sequences [64].

To measure and analyze variation and composition of microbial communities, indices describing diversity have been implemented. In 1960, alpha- and beta diversity were defined, where the alpha diversity allows to estimate species number (richness) and distribution (evenness) within a particular sample, while a beta diversity measure acts like a similarity score between populations of different samples [65]. Since then, several different diversity indices have been defined [66]. Among the most commonly used diversity indices are taxon based approaches, Simpson's index [67], Coverage (C) [68], Chao1 richness estimator [69], Shannon index [70] and Shared OTUs [71–73]. To date, at least 15 different tools for taxonomic profiling are available for metagenomics, already compared and benchmarked by use of various datasets [60].

Current understanding of the equine microbiome

For all mammalian species, scientific evidence points towards a strong relationship between enteral microbiome composition and its function [74]. Considering data available on composition of microbial communities residing in different animal species' guts, current knowledge exposes a clustered gastrointestinal microbiome according to differences in their gut microbiota for all carnivores, herbivores and omnivores [75]. For instance, nourishment based on animal proteins results in an increased number of *Firmicutes* among the respective microbiota while, in contrast, plant based diets result in more fibers and those microbiomes yield an increased number of *Bacteroidetes*, cellulose- and xylan degrading bacteria [28]. Recent studies revealed distinct individual ecosystems for each compartment of the equine gut, with more similarities regarding composition of microbiota in neighboring compartments than between more distant ones [30]. At present, two main regions need to be distinguished: the upper- and the lower GIT [29]. By way of comparison, the upper equine gut (stomach, jejunum and ileum) shows a more variable microbiota substantiated due to a high throughput of environmental bacteria present in the forage. Moreover, members of the α-*Proteobacteria* such as *Methylobacterium* sp., *Rhizobium* sp. and *Sphingomonas* sp. are commonly abundant in this gut region [29]. In contrast, composition of the microbiota residing in the lower GIT of horses (caecum and colon) seems remarkably stable, despite variables such as individual history, breed or age.

Beside a rich population including a diverse spectrum of bacterial species with their bacteriophages, the equine hindgut microbiota also encompasses protozoa, fungi, yeasts, and archaea [76]. Considering resident bacteria, *Firmicutes*, *Bacteroidetes* and *Verrucomicrobia* are amongst the predominating phyla in the equine hindgut [28, 30, 77–79]. Further studies revealed an abundant population of methanogenic archaea in the equine colon

[76]. These microbes metabolize H_2 and CO_2 to produce methane [80] and probably support the degradation of cellulolytic bacteria in the lower gut [81, 82]. Metabolic pathways essential for sufficient nourishment of horses depend on functional interactions of mandatory microbes needed for a successful degradation of nutrients. Some bacterial families belonging to the resident phyla as well as other microorganisms of the equine GIT have been characterized with respect to their (predicted) nourishment-associated activity (Table 1).

Activity of microorganisms leading to changes within gastrointestinal microbiota in horses. Further proposed effects of distinct microorganisms are indicated by small letters. Abbreviations: Ref., Reference; [a], generates neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) [83]; [b], associated with succinate pathway for production of short chain fatty acid propionate [84]; [c], butyrate producers [85], butyrate shows protective function for colonocytes [86], [d], major producers of short chain fatty acids [87]; [e], possesses coding region for major exoglucanase [88]; [f], use of H_2 and CO_2 to produce methane, might boost the carbohydrate-degrading activity of cellulolytic bacteria [80, 81].

An important role in the enteral degradation of vegetal fibres was assumed for anaerobic fungi. In 2003, *Piromyces equi*, an anaerobic monocentric fungus, was reported to possess a major exoglucanase, which is fully capable of digesting cellulose [88, 89]. Next to *Piromyces equi* only two other morphological and metabolically different fungal species were described: *Piromyces citronii* and *Caecomyces equi* [9]. Evidence also exists for other novel fungal taxa grown from equine feces, which still need to be characterized and investigated further [90].

At present, knowledge is scarce concerning the role of bacteriophages in the equine gut. Several studies estimate a proportion of 10^{10} to 10^{11} bacteriophages per gram feces [91, 92], including up to 60 morphologically distinct phage types [93]. Golomidova et al. (2007) provided evidence of phage affinity for bacteria with high population numbers [92]. A dense population is commonly more embedded and adjusted in its biological environment than bacteria with a lower population number. The authors pointed out a direct link between diversity and abundance of *Escherichia coli* strains and the relative abundance of specific coliphages. Many ecological systems are shaped from predator-prey interactions. However, the GIT often promotes commensal relationships between different members of the community [94]. It is assumed that bacteriophages influence the fitness of intestinal bacteria and support colonization and host adaption, particularly in cases of environmental changes, including antibiotic forces [94–96]. Amongst others, Cann et al. have identified *Siphoviridae*, *Myoviridae*, *Podoviridae* and vertebrate *Orthopoxvirus* in horse feces, but 26% of viruses identified in that study were unclassified in 2005 [91].

Yet, the role of intestinal protozoa such as *Ciliates* [97, 98] is not well understood. A beneficial while only limited function in cellulose digestion and degradation of pectin seems likely [99, 100].

Age is among the most influencing factors of individual enteral microbiomes, while the initial microbiome already depends on the location of birth. In humans, even the type of birth (natural delivery or *sectio caesarea*) brings about differences with respect to initial microbiome composition [101].

While new born foals commonly have a rich and diverse microbiota with *Firmicutes* as predominant phyla [102, 103], foals between two and 30 days in comparison host a decreased level of different microorganisms, with *Verrucomicrobia* (e.g. *Akkermansia* spp.) predominating [102]. After 60 days, the microbiome consists of a relatively stable population, and microbiomes of 9 month-old foals only show few differences compared with those of adult individuals [102]. Considering levels of species diversity, microbiomes of older horses (19–28 years) once again show a decreased level with respect to the diversity of residing organisms [28]. A comprehensive overview about factors affecting GIT microbiome composition while affecting relative abundance of distinct microorganisms in horses is given in Table 2. Interestingly, the degree of domestication of *Equidae* under consideration seems to have an important impact on their enteral microbiome, which is summarized in Fig. 2. Free living individuals show a more diverse microbiome composition as their conspecifics in captivity [101], an observation which might mirror loss of diversity among human enteral microbiomes in more industrialized countries [104]. Horse domestication interferes with social structures like inter-individual relationships, shared environments and nourishment [101]. Comparative composition analysis of microbiomes of non-domesticated and domesticated horses living in the same area with similar plant diets revealed that fecal microbiomes of the latter group had a significantly lower abundance of the Clostridia genus *Phascolarctobacterium* for producing the short chain fatty acid propionate [101]. Moreover, microbiomes of non-domesticated horses harbor a significantly higher relative abundance of producers of enteric methane like *Methanocorpusculum archaea* [101], which may boost the carbohydrate-degrading activity of cellulolytic bacteria (Table 2).

Attempts to define the "core bacteria" of the equine microbiome

Microbial communities which commonly appear in all assemblages associated with a specific habitat are likely critical to the function of that environment [36]. Consequently, identifying of a defined core composition of microorganisms is an important step in defining a 'healthy'

Table 2 Effects of specific factors on equine intestinal organism abundances

Factor	Effect on organism abundance	Organisms in enteral microbiome	Reference
highly concentrated (grain) feed	increase	lactic acid bacteria, especially *Streptococcus spp.* and *Lactobacillus spp.*	[181] [31]
high-starch fed	increase	*Succinivibrio*	[28]
high-starch fed	decrease	*Clostridiales, Lachnospiraceae*	[28]
haylage	putative increase	*Fibrobacter succinogenes, Fibrobacter intestinalis*	[106]
grass-based diet	increase	*Bacteroidetes, Lachnospiraceae Bacillus, Lactobacillus, Streptococcus*	[181]
grass-based diet	decrease	*Fibrobacter, Ruminococcus*	[181]
high oil and high starch diets	increase	*Proteobacteria*	[28]
increasing age	increase	*Euryarchaeota, Actinobacteria, Bacteroidetes, Chlamydiae, Chloroflexi, Planctomycetes, Spirochaetes,* TM7, *Verrucomicrobia*	[182]
increasing age	decrease	*Proteobacteria, Gammaproteobacteria, Enterobacteriaceae, Enterococcus*	[182]
domestication	lower	*Methanocorpusculum*	[101]
pH below 6.0	decrease	*Ruminococcus albus, Fibrobacter succinogenes*	[6]
pH below 6.0	increase	*Streptococcus bovis, Lactobacillus spp., Mitzuokella spp.*	[6]
parasite egg burden	decrease	*Bacteroides, Clostridium XIVa, Ruminococcus, unclassified Lachnospiraceae*	[178, 183]
parasite egg burden	increase	*Clostridium* IV, *Coprococcus, Anaerovibrio, Agreia, Oscillibacter, Turicibacer,* unclassified *Cystobacteraceae, Campylobacter, Bacillus, Pseudomonas*	[178, 183]
laminitis	increase	*Lactobacilli, Escherichia coli*	[138, 184]

microbial community [36]. The core community at the Operational taxonomic Unit (OTU) level in feces is defined as "being present in all samples included in the study at 0.1% relative abundance (or greater)" [105]. Defining an essential core might be useful to predict the impact of perturbations and to preserve or restore a microbiome associated with a healthy condition [36]. Despite the unarguable individual composition of each horses' microbiome [106] a so called "core microbiome" was declared including "key microbes" present in most individuals [7, 28, 30, 35]. Considering the vast diversity of intestinal bacteria known for ruminants, the equine gut microbiome seems to comprise a lower number of species as "core" population, with the richest diversity (33 bacterial families) residing in the right dorsal colon [7].

Firmicutes represent the largest phylum of the equine intestinal bacterial community ranging from 40% up to 90% in different compartments, including *Clostridia* and *Bacilli* [7, 30]. *Clostridiales* such as the aerobic *Lachnospiraceae* seem to be a part of the intestinal "core microbiome" in all mammals [28]. They produce butyrate which is known for its protective function of colonocytes [107]. Although the families *Ruminococcaceae* and *Fibrobacteraceae* represent only a small percentage of the bacterial community, both were considered as being part of the "core" along the entire equine hindgut [9]. These bacteria are involved in plant-wall degradation (Table 1) and their absence may influence the overall balance of the microbiome, therefore these cellulolytic bacteria were seen as "keystone species" [108].

The second largest group to address here are *Proteobacteria*, comprising a broad range of gram-negative bacteria, including Enterobacteriales and Pseudomonadales. The intestinal diversity of *Proteobacteria* is driven by the uptake from the environment, where these bacteria reside to certain abundances. Consistently, *Proteobacteria* are predominant in the upper part of the equine GIT [29], with highest abundance in the equine Ileum (including *Pasteurellales*) with approximately 33% [30]. In view of the overall diversity of residing *Proteobacteria*, various functional activities can be assumed, which are not entirely known yet. For instance, some members of *Proteobacteria* are known for their role in intestinal nitrogen fixation [109]. Nevertheless, an overabundance is reported to be associated with inflammatory intestinal diseases and dysbiosis like colic in horses [25, 110].

The third group consists of *Verrucomicrobia*. *Verrucomicrobia* is an abundant phylum within the environment, especially in soil [111]. *Verrucomicrobia* are part of the PVC superphylum, named for its member phyla *Planctomycetes*, *Verrucomicrobia* and *Chlamydiae, which* are distinct phyla of the domain bacteria proposed initially on the basis of 16S rRNA gene sequence analysis [112]. These bacteria are considerable residents in equine caecum, small colon, rectum and feces with relative abundance ranging from 10 to 23% [30]. *Verrucomicrobia* gained

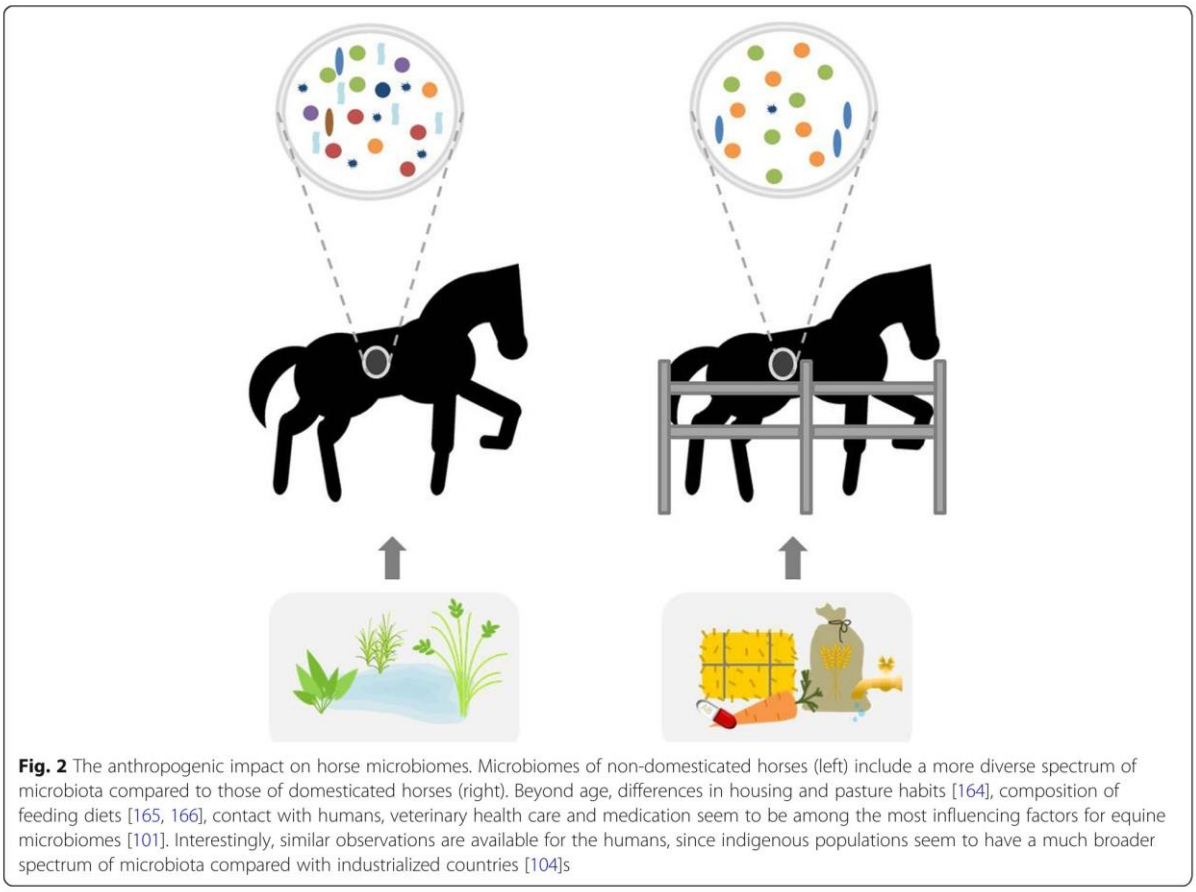

Fig. 2 The anthropogenic impact on horse microbiomes. Microbiomes of non-domesticated horses (left) include a more diverse spectrum of microbiota compared to those of domesticated horses (right). Beyond age, differences in housing and pasture habits [164], composition of feeding diets [165, 166], contact with humans, veterinary health care and medication seem to be among the most influencing factors for equine microbiomes [101]. Interestingly, similar observations are available for the humans, since indigenous populations seem to have a much broader spectrum of microbiota compared with industrialized countries [104]s

increasing attention in obesity and metabolic disease research in humans [113, 114]. Akkermansia, a mucin-degrading genus within the phylum Verrucomicrobia helps to maintain the integrity of the mucin layer and decreases bowel inflammation [115]. In summary, the overall diversity of the core bacterial community of domesticated horses seems to be surprisingly low, a fact that was discussed as a possible reason for the sensitivity of horses to GIT diseases [28].

Diseases, drugs and feeding are associated with changes in the equine microbiome

Horses have a sensitive intestinal tract, and exercise [10], transport and fasting [38] ensure verifiable changes in the equine microbiome composition. A comprehensive overview on studies addressing composition and changes of the equine microbiome in healthy and diseased animals together with the techniques used by the individual study group is provided in Additional file 1. Important findings from these studies addressing major issues of microbiome research in horses will be explained and summarized in the following section.

Since an appropriate and balanced diet is essential for optimal successful degradation of nutrients and health in Equidae, incorrect feeding might induce dysbiosis or increases general vulnerability [31, 116]. Dysbiosis in microbiome composition was found to be associated with horses suffering from enteral disorders [25, 110].

A balanced system of intestinal microorganisms is an important health value, not surprising an unbalanced enteric microbiota could cause colitis [25]. Colitis refers to an inflammation of the gut mucosa of the large bowel (cecum and colon) which is either characterized by an acute or long-term process. Commonly, acute colitis is characterized by a sudden onset of profuse watery diarrhea. The fast and excessive loss of enteric fluids is able to induce death by dehydration or even hypovolemic shock [117]. Equine colitis can be triggered by multiple conditions including bacterial infections, infestation by parasites or antimicrobial treatment [117–119]. Bacteria-associated inflammation is commonly associated with *Salmonella* species, *Clostridioides difficile*, *Clostridium perfringens* and *Neorickettsia risticii* (Potomac horse fever) [120]. *Fusobacteria,* commonly rare in healthy

horses, seem to be significantly enriched in case of diarrhea and colitis [25, 121]. Additionally, foals with diarrhea have shown a less rich microbiome composition in comparison with healthy foals together with decreased abundances for *Lachnospiraceae* and *Ruminococcaceae* [122].

It is difficult to pinpoint a precise cause for gut inflammation since further variables such as age, living space and individual case history of the horse influence the entire community of residing microbiota [117]. A common non-infectious cause of colitis in horses is receiving antimicrobials. Many reports have shown the association between antimicrobial treatment of horses and colitis [123, 124]. An imbalance of the fragile equine intestinal microbiota which may lead to bacterial overgrow seems to be inducible by a lot of antibiotics, including Penicillin [125], Cephalosporins [126] or Fluoroquinolones [127]. These antimicrobials have been associated with equine colitis [128], reflected by a significant transformation of the equine microbiome structure after consumption [37]. Costa et al. (2015) reported changes of equine fecal microbiota induced by trimethoprim-sulfadiazine, emphasizing a significant decrease of bacterial richness and diversity together with a drastic decrease of endosymbionts such as *Verrucomicrobia* [37]. Changes in the equine microbiome composition induced by antibiotics seemed to be specific for each drug and might therefore be predictable [37]. It seems to take 25 days to re-build the microbial composition back to individual baseline levels, but differences are still detectable beyond that time [37].

Moreover antimicrobial therapy is among the main risk factors for *Clostridioides difficile* associated colitis and colonization not only in humans but also in horses and other companion animals like dogs and cats [125, 129, 130]. Disruption of host microbiota homeostasis with reduction of microbiota density is most likely associated with reduced colonization resistance and may also contribute to a pro-inflammatory host immune response [131].

Colic is one of the most lethal diagnoses for horses which only 63% will survive [132]. Besides sand ingestion and colon displacement [117], further (stress) factors can be responsible for colic. Changes in feeding routine are also under suspicion for inducing rapid shifts in microbiome composition [133] and increased risk for colic [10, 134]. To identify microbiome changes strongly associated with colic [135], physiological changes in microbiomes of healthy horses need to be explored [106]. At present, there is a lack of data addressing the role of particular microbiome changes for the development of the equine colic syndrome.

Receiving anesthesia seems to be a putative further factor able to cause changes of the equine microbiome structure. Shifts on genus level were reported for horses under anesthetic for six hours, including an enrichment of the genera *Anaerostipes*, *Ethanoligenens* and *Enterococcus* (*Firmicutes*) 24 h later, while an enrichment of *Ruminococcus* (*Firmicutes*) was recorded after 48 h. However, further research is needed to gain more insights into anesthesia and its putative power to induce shifts within the equine intestinal microbiome.

Rapid proliferation of lactic acid producing bacteria is a feared consequence of a high starch diets, promoting lactic acidosis which is often followed by laminitis [136]. Interestingly laminitis was assumed to be associated with proliferation of streptococci [76], since earlier studies reported co-incidence [137, 138].

Use of probiotics and their effects in horses

Recently, products classified as "probiotics" have reached the commercial market, not only for humans but also for horses. In 2001, experts of the World Health Organization (WHO) and Food and Agriculture Organization of the United Nations and the WHO (FAO/WHO) provided a very useful and actual definition of a probiotic: "live strains of strictly selected microorganisms which, when administered in adequate amounts, confer a health benefit on the host [139, 140]". In the US, probiotics can either be classified as a drug needed to gain approval from the Food and Drug Authority (FDA) or as a feed supplement "generally regarded as safe (GRAS)" based on information provided by the producers, so they do not need to go through FDA approval [141]. In the European Union (EU), probiotics are regarded as feed additives and gut flora stabilizers for healthy animals [33]. The EU applies very strict regulations for products labeled as probiotics. Producers need to prove product identity, safety and efficacy to a scientific committee. Assessment and approval from the scientific committee and authorization under EU council regulation (EC) no. 1831/2003 on additives for use in animal nutrition is needed before market introduction [142]. In 2008, the EC no. 429/2008 provided detailed rules for the implementation of regulation 1831/2003. So far, bacteria such as *Lactobacillus*, *Enterococcus*, *Bacillus*, *Streptococcus* and *Bifidobacterium* are considered as putative beneficial probiotics for horses [141]. Probiotics should be able to survive the extreme gastric environment, have an antimicrobial property against pathogens and adhere to mucus and epithelial cells [143]. Probiotics for horses are designed to reach and establish themselves in the large colon, were many diseases occur. A recent study investigated the effects of multi-strain probiotics on the bacterial microbiota of foals during and after administration [144]. Limited changes were only found concerning relative abundance of bacterial families, with an enrichment of *Lactobacillus* in the probiotic group at week six [144]. Yet, evidence of probiotic efficiency in horses is weak despite

several putative clinical applications including acute enterocolitis [145], diarrhea in foals [146] as well as fecal sand clearance [147].

Future perspectives

Although microbiome research is considered an emerging science, with some areas of research still in their infancy, the field is progressing rapidly [148]. Nowadays, the most important research task is to gain a deeper understanding of the complex relationships between the gut microbiota, well-being and disease [149]. A meta-analysis of gut microbiome studies in humans revealed that some diseases are marked by the presence of potentially pathogenic microbes, whereas others are characterized by a depletion of health-associated bacteria [150]. Only recently, the first study investigating changes in the fecal microbiota using 16S rRNA gene data from microbiome analysis over a prolonged period (52 weeks) of healthy horses was published [106]. Throughout all seasons, *Firmicutes* and *Bacteroidetes* dominated the fecal microbiota, but supplementary forage, season and ambient weather conditions were significantly associated with change in the fecal microbiota composition [106]. These data provide an excellent starting point for further microbiome research investigating changes associated with metabolic disorders, infectious diseases or effects of drugs, since the first framework for a microbial composition associated with healthy horses has been set. However, disturbance of gut microbiota leading to or indicating illness still needs to be defined more precisely for horses.

Similar to the current trends in human medicine it might be possible to develop individual treatment opportunities for certain kinds of equine diseases which were marked through a certain and distinct pattern of microbial composition like equine grass sickness, laminitis or colitis. Moreover, fecal transplants are used to treat intestinal disorders including inflammatory bowel disease and recurrent *Clostridioides difficile* infections, and may eventually be used to treat a long list of disorders [151]. Besides technical questions associated with data generation and analysis, further research is needed to address the benefits and limits of different sampling sites for microbiome research in horses. Representativeness of different GIT sampling sites and feces have been discussed before, for example in pigs [152, 153]. A recent study on free-ranging bats revealed that the diversity and composition of intestine and guano samples differed substantially, likely reflecting the distinct processes that are known to occur in these microhabitats [154], as described above for different parts of the GIT in horses. Moreover, fecal samples retained more signal of host diet than intestinal samples, suggesting that fecal and intestinal sampling methods are not interchangeable [154].

As a further future perspective, research focused on effects of different antibiotics and/or application routes on the equine microbiome might reveal whether the absence or presence of certain key microbes is associated with drug-induced colitis. Currently, multi-drug resistance (MDR) in zoonotic bacteria such as *Escherichia coli* and *Staphylococcus aureus* are still a rising issue in equine medicine [155, 156]. Thus, further research might also identify dosages and application intervals for antibiotics which were not beneficial and sufficient for the horse patient alone, but also associated with a low selective pressure on resistant bacterial variants and thus hinder further accumulation of zoonotic MDR in horse clinics. In addition, metagenomics is currently considered as the most straightforward and affordable data that can be used to track transmission of strains [151], providing new perspectives to follow transmission routes of zoonotic bacteria.

Conclusion

Our review summarizes the current understanding and progress in equine microbiome research (Additional file 1), which clearly is not yet at eyelevel with the latest vast progress in human medicine. Nonetheless, important first research initiatives have been kicked off, and fields worth investigating have been addressed clearly. Our review provides insights in commonly used techniques to explore the equine microbiome, their benefit and limitation as well as tools for data analysis. A smart combination of different techniques including the wet lab (Fig. 1) appears to be a good strategy to broaden and sustain the research outcomes.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s42523-019-0013-3.

Additional file 1: Overview on horse microbiome surveys

Abbreviations

Bp: Base pair; EC: Council regulation; EU: European Union; FAO: Food and Agriculture Organization; FDA: Food and Drug Authority; GIT: Gastrointestinal tract; MALDI-TOF-MS: Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry; MDR: Multi-drug resistance; NGS: Next-generation sequencing (NGS); OUT: OPERATIONAL Taxonomic Units; QIIME: Quantitative Insights into Microbial Ecology; rRNA: Ribosomal ribonucleic acid; USEAR: Ultra-fast sequence analysis; WGS: Whole genome sequencing; WHO: World Health Organization

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Authors' contributions

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4.2. **Veröffentlichung II**

Frequency, local dynamics and genomic characteristics of ESBL-producing Escherichia coli isolated from specimens of hospitalized horses

Anne Kauter, Lennard Epping, Fereshteh Ghazisaeedi, Antina Lübke-Becker, Silver A. Wolf, Dania Kannapin, Sabita D. Stoeckle, Torsten Semmler, Sebastian Günther, Heidrun Gehlen, Birgit Walther

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Beiträge der Autoren

BW, AL-B und HG entwarfen das Projekt. SS, HG und BW planten und gestalteten die Experimente. TS sequenzierte die Isolate. AK, DK, SS, HG und AL-B führten die Laboranalysen durch. BW, LE, AL-B, SW, TS, SG und FG analysierten die Daten. AK, SW und BW schrieben die Veröffentlichung.

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Frequency, Local Dynamics, and Genomic Characteristics of ESBL-Producing *Escherichia coli* Isolated From Specimens of Hospitalized Horses

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Previous research identified veterinary clinics as hotspots with respect to accumulation and spread of multidrug resistant extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* (EC). Therefore, promoting the prudent use of antibiotics to decrease selective pressure in that particular clinical environment is preferable to enhance biosecurity for animal patients and hospital staff. Accordingly, this study comparatively investigated the impact of two distinct perioperative antibiotic prophylaxis (PAP) regimens (short-term versus prolonged) on ESBL-EC carriage of horses subjected to colic surgery. While all horses received a combination of penicillin/gentamicin (P/G) as PAP, they were assigned to either the "single-shot group" (SSG) or the conventional "5-day group" (5DG). Fecal samples collected on arrival (t_0), on the 3rd (t_1) and on the 10th day after surgery (t_2) were screened for ESBL-EC. All isolates were further investigated using whole genome sequences. In total, 81 of 98 horses met the inclusion criteria for this study. ESBL-EC identified in samples available at t_0, t_1 and t_2 were 4.8% (SSG) and 9.7% (5DG), 37% (SSG) and 47.2% (5DG) as well as 55.6% (SSG) and 56.8% (5DG), respectively. Regardless of the P/G PAP regimen, horses were 9.12 times (95% CI 2.79–29.7) more likely to carry ESBL-EC at t_1 compared to t_0 ($p < 0.001$) and 15.64 times (95% CI 4.57–53.55) more likely to carry ESBL-EC at t_2 compared to t_0 ($p < 0.001$). ESBL-EC belonging to sequence type (ST) 10, ST86, ST641, and ST410 were the most prevalent lineages, with $bla_{CTX-M-1}$ (60%) being the dominant ESBL gene. A close spatio-temporal relationship between isolates sharing a particular ST was revealed by genome analysis, strongly indicating local spread. Consequently, hospitalization itself has a strong impact on ESBL-EC isolation rates in horses, possibly masking differences between distinct PAP regimens. The results of this study reveal accumulation and spread of multi-drug resistant ESBL-EC among

horses subjected to colic surgery with different P/G PAP regimens, challenging the local hygiene management system and work-place safety of veterinary staff. Moreover, the predominance of particular ESBL-EC lineages in clinics providing health care for horses needs further investigation.

Keywords: horse, ESBL, *Escherichia coli*, antibiotic resistance, multidrug resistant, spread

INTRODUCTION

The occurrence of zoonotic and multidrug resistant (MDR) pathogens, such as methicillin resistant *Staphylococcus aureus* (MRSA), *Acinetobacter* spp. and extended-spectrum β-lactamase (ESBL)-producing Enterobacterales, are an ongoing challenge to both, biosecurity and hygiene in veterinary clinics (Walther et al., 2017, 2018a; Royden et al., 2019). Previous studies reported the constant admission of equine patients carrying ESBL-producing *Escherichia coli* (ESBL-EC) to horse clinics (Apostolakos et al., 2017; Walther et al., 2018b). Moreover, hospital associated infections (HAI) have been linked to local spread of the above-mentioned MDR pathogens in horse clinics (Walther et al., 2014b; van Spijk et al., 2019). These infections are often difficult to handle due to a multitude of additional antimicrobial resistances (AMR) commonly associated with ESBL-EC (Wieler et al., 2011). In recent years, reports on ESBL-EC carriage and fecal shedding in horses have increased (de Lagarde et al., 2019; Hordijk et al., 2020). Hospital stay alone, for instance, as well as antibiotic courses have only recently been identified as important risk factors for ESBL-EC colonization of equine patients (Schoster et al., 2020).

So far, knowledge on ESBL-EC isolation rates and local dynamics considering particular equine patient groups, i.e., hospitalized horses subjected to colic surgery, is scarce. A combination of penicillin and gentamicin (P/G) is among the most common medications for perioperative antibiotic prophylaxis (PAP) in horse surgery, including operative abdominal (colic) interventions (Dallap Schaer et al., 2012; Teschner et al., 2015). While different guidelines for antibiotic administration in horses strongly suggest to abstain from PAP prolongation beyond 24 h after elective interventions (Weese et al., 2008; Swedish Veterinary Association, 2013), horses subjected to abdominal surgery seem to commonly receive the P/G combination for 5 days afterward (Teschner et al., 2015). In a previous study, reduction of the P/G PAP to 72 h (3 days) after surgery showed no significant influence on the clinical outcome of the equine patients (Dallap Schaer et al., 2012). However, the putative effect of decreased selective (antibiotic) pressure on the local MDR pathogen load, especially ESBL-EC, was not investigated in the mentioned study.

This study was carried out within an interdisciplinary research network (Köck et al., 2020) investigating strategies to enhance the prudent use of antibiotics in veterinary medicine. Accordingly, the aims were (i) to assess ESBL-EC phenotypes and isolation rates in hospitalized horses, (ii) to investigate whether a short-term or a prolonged P/G PAP influences these rates, and (iii) to identify potential ESBL-EC transmission events and local dynamics.

MATERIALS AND METHODS

Ethics and Study Outline

According to the German regulation authorities for research with animal subjects, the comparison of two PAP regimens does not require approval (Landesamt für Gesundheit und Soziales, Berlin, 18.04.2017). Written owner's consent with respect to involvement of their horses in the study was obtained directly during the hospital admission process.

Here, we have comparatively investigated the effect of two distinct PAP regimes on isolation rates of ESBL-EC from specimens of horses subjected to colic surgery. A graphical abstract of the study design is provided in **Figure 1**. Horses included in this study were assigned per lot (sealed envelope drawn at the day of surgery) to one of two groups: the "5-day group" (5DG) which received a combination of penicillin (22,000 IU/kg q 6 h) and gentamicin (6.6 mg/kg q 24 h) (short: P/G) (Dallap Schaer et al., 2012; Teschner et al., 2015) for 5 days and the "single-shot group" (SSG), which received P/G PAP only directly before, and, if necessary due to prolonged surgery- also during the elective procedure Briefly, horses considered as study participants needed to be older than 1 year, had to be clear of any clinical signs of infectious diseases before the intervention and had to recover from anaesthesia ("stand up") after surgery. Furthermore, equine patients were excluded from further participation (i.e., consideration of specimens at t_1 and/or t_2; **Figure 1**) when their hospital stay ended prematurely due to euthanasia (premature), discharge and/or if the antibiotic regime they had been assigned to was no longer strictly followed, regardless of the particular reasons for these interventions. In consequence, the number of valid specimens available per time point shrank toward the end of the study.

Sample Collection

In order to investigate ESBL-EC rates in specimens from the equine patients, fecal samples and nostril swabs (Copan Liquid Amies Elution Swab, flocked) were directly collected from each horse at the time of hospital admission (t_0) as described previously (Walther et al., 2018b). A second and a third sampling was carried out on day three (t_1) and ten (t_2) after surgery (**Figure 1**). All specimens were stored at 4°C and subjected to microbiological diagnostics latest on the next day. We included the nostril sampling in this study, since dust particles containing ESBL-EC have been reported as a potential source of contamination and, possibly, colonization in farmers exposed to an environment prone to MDR pathogens (Fischer et al., 2017).

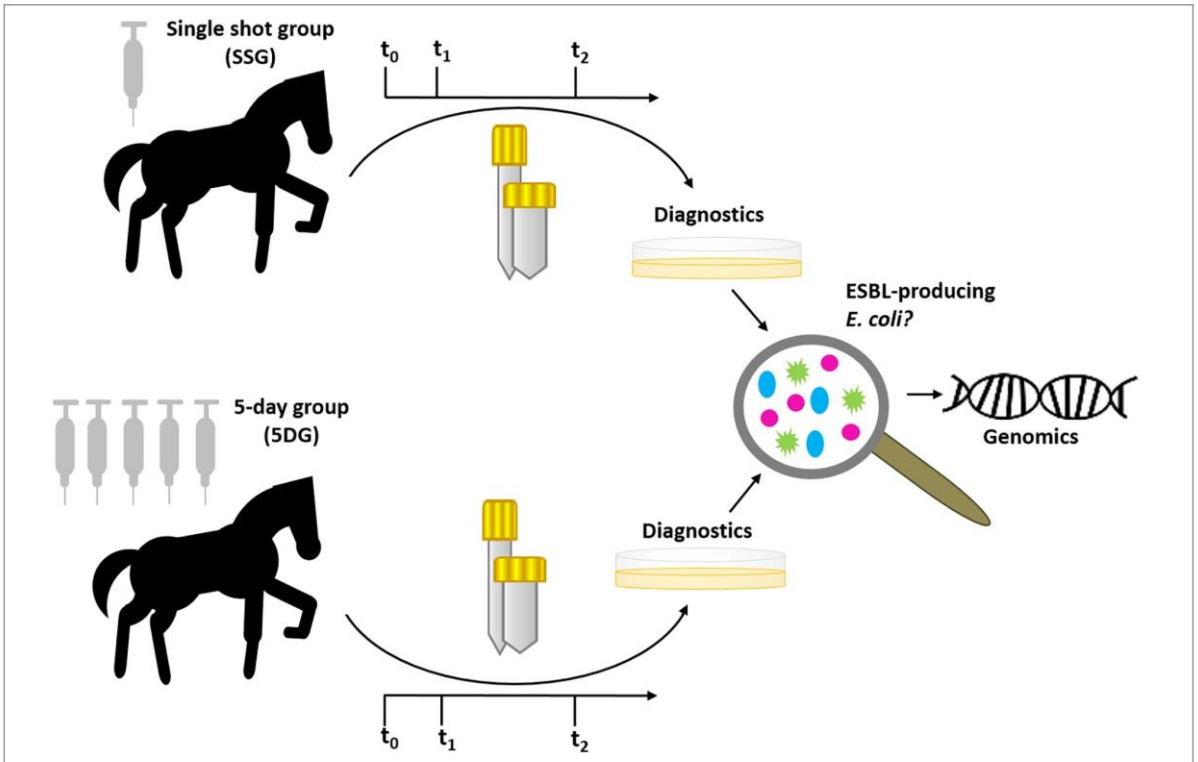

FIGURE 1 | Graphical abstract of the prospective study. Horses were assigned to either the "single-shot group" (SSG) or the "5-day group" (5DG) per lot (sealed envelope drawn directly at hospital admission). Fecal samples and nostril swabs were collected at t_0 (hospital admission), t_1 (3 days after surgery) and t_2 (10 days after surgery). All samples were screened for putative ESBL-EC, subjected to the confirmatory testing followed by AST and whole genome sequencing.

Bacterial Identification and Antimicrobial Susceptibility Testing

All fecal samples and nostril swabs were initially cultured on Brilliance™ ESBL Agar plates (Thermo Scientific™, Germany) overnight. Colonies showing characteristic growth signatures of ESBL-EC on chromogenic screening plates were further investigated. In case of distinct phenotype appearances of presumptive EC growing on the plates, all isolates were subjected to the ESBL confirmatory test according to the Clinical and Laboratory Standards Institute (CLSI) recommendations (Clinical and Laboratory Standards Institute [CLSI], 2018). Additionally, disk diffusion method was performed to detect putative plasmidal AmpC (pAmpC)-producers using cefoxitin (30 μ) with a ≤18 mm screening cut-off (Polsfuss et al., 2011). Species confirmation was achieved by Matrix-Assisted Laser Desorption/Ionization-Time Of Flight (MALDI-TOF) mass spectrometry (Bruker, Germany). The Vitek2 AST-GN38 and AST-GN 96 cards (bioMérieux, Germany) were used for antimicrobial susceptibility testing (AST) on the VITEK®2 system (BioMérieux, Germany) including enrofloxacin, gentamicin, trimethoprim-sulfamethoxazole and tetracycline following CLSI standards (Clinical and Laboratory Standards Institute [CLSI], 2020).

Whole Genome Sequencing, Antimicrobial Resistance Gene Detection and Phylogenetic Relationship of the Isolates

DNA isolation was performed for all confirmed ESBL/pAmpC-EC with the QIAamp® DNA Mini Kit (250) (Qiagen, United States) and the respective DNA was subsequently stored at −20°C. Whole genome sequencing (WGS) was performed using Illumina MiSeq 250 bp paired-end sequencing with an obtained coverage >80. After adapter trimming, 247.9 bp remained on average per read. Illumina raw read data sequenced for this study is available at National Center for Biotechnology Information (NCBI) under Bioproject ID: PRJNA698802.

Assembled draft genomes of the isolates were obtained from SPAdes v.3.13.1 (Bankevich et al., 2012) and annotated with Prokka v.1.14.6 (Seemann, 2014). WGS data were used for the determination of the sequence type (ST) and resistance genes (threshold: 98% ID, 90% minimum query coverage) performed by Center for Genomic Epidemiology (Bortolaia et al., 2020). PlasmidFinder and pMLST v.1.3 pipelines were used for the identification of plasmids associated with Enterobacteriales (Zankari et al., 2012; Carattoli et al., 2014). A core genome alignment was calculated using Roary v.3.13.0 (Page et al., 2015),

with a minimum sequence identity of 95%. The alignment was used to construct a maximum likelihood-based phylogeny with RAxML v.8.2.10 (Stamatakis, 2014) and 100 bootstraps under the assumption of the gtr-gamma DNA substitution model.

In order to investigate potential MDR pathogen transmission events within the horse clinic, pairwise single-nucleotide polymorphism (SNP) distances were calculated with snp-dists v.0.7.0[1] and visualized using the R package "pheatmap"[2]. Furthermore, pairwise SNP distances were utilized to identify clonally related ESBL-EC genomes for each of the major sequence type complexes (STC) identified (**Supplementary Figures 1–3**). Since the mutation rate for ESBL-EC genomes was estimated to be 4.14×10^{-7} SNPs per site per year (Ludden et al., 2020), a clonal relationship was defined conservatively as isolates with five or fewer SNPs between any two members of a group, as reported previously (D'Souza et al., 2019).

Statistical Analysis

Statistical analyses were conducted using the SPSS software (Statistical Package of Social Science, version 26.0, Chicago, IL, United States). The comparative analysis of the proportion of ESBL-EC positive equine patients was performed using the McNemar test for categorial variables and by analyzing the proportions of ESBL-positive fecal samples at two sampling time points (t_0 and t_1) or (t_0 and t_2). Generalized estimating equation (GEE) analysis for clustered binomial responses was used to determine the effect of hospitalization (t_1 and t_2), different P/G PAP regimes (short-term or prolonged), and a potential interaction effect, on the occurrence of ESBL-EC in fecal samples of horses subjected to colic surgery. A p-value of < 0.05 was considered statistically significant.

RESULTS

Study Population and Sample Acquisition

A total of 98 horses subjected to abdominal surgery due to acute abdomen (colic) from January 2018 to February 2020 have been considered as study participants. However, only 81 of these horses met the inclusion criteria for this study at t_0, with $n = 32$ belonging to the SSG and $n = 49$ to the 5DG (**Figure 2**). Due to the medical nature of colic syndrome, which is often accompanied by defecation disorders, samples were not available for all horses at all sampling times, as indicated by the numbers associated with the sample symbols in **Figure 2**. Moreover, until the end of the study, samples from five horses belonging to the SSG and 12 from the 5DG were excluded from further evaluation—the reasons included sudden discharge, euthanasia due to animal welfare or deviation from the antibiotic protocol. In addition, temporary unavailability of Benzylpenicillin for veterinary use caused premature termination of the study in 02/2020. This event lead to a restricted number of study participants and therefore also limited the representativeness of the study outcome.

[1]https://github.com/tseemann/snp-dists

[2]https://github.com/raivokolde/pheatmap

Isolation Rates of ESBL-EC in Specimens of Equine Patients Subjected to Colic Surgery Receiving Different Regimes of P/G PAP

Considering the isolation rates for each sampling point, ESBL-EC were occasionally identified in nostril swabs obtained from both horse groups investigated (**Figure 2** and **Supplementary Table 1**). In total, 2/87 (2%) nostril swabs obtained from the SSG and 8/129 (6%) of the 5DG were ESBL-EC positive.

In the SSG, 1/21 (4.8%) fecal samples collected at the time of hospital admission (t_0) were positive for ESBL-EC, while the isolation rate increased to 10/27 (37%) at t_1. At the tenth day of hospital stay, 15/27 (55.6%) of the SSG fecal samples were positive for ESBL-EC ($p = 0.003$). The ESBL-EC rate among samples from horses belonging to the 5DG was 3/31 (9.7%) at t_0 and increased to 17/36 (47.2%) at t_1. At t_2 (i.e., the tenth day after surgery and the fifth day after the last antibiotic course), 21/37 (56.8%) fecal samples were ESBL-EC positive ($p = 0.013$; **Figure 2**). There were no statistically significant differences between the proportions of ESBL-EC positive fecal samples of the two study groups investigated (**Figure 2**). Regardless of the P/G PAP regime, horses were 9.12 times (95% CI 2.79–29.7) more likely to carry ESBL-EC at t_1 compared to t_0 ($p < 0.001$) and 15.64 times (95% CI 4.57–53.55) more likely to carry ESBL-EC at t_2 compared to t_0 ($p < 0.001$).

Antibiotic Susceptibility Profiles and Antimicrobial Resistance Genes

AST results for the most common antibiotics in equine medicine (enrofloxacin, gentamicin, trimethoprim-sulfamethoxazole and tetracycline) obtained for 85 ESBL-EC are shown in **Table 1**. Of note, 75 (88%) of the 85 investigated ESBL-EC isolates showed resistance toward aminoglycoside gentamicin and 78 (92%) toward the sulfonamide-trimethoprim combination. Eight isolates were ESBL-producers only, two isolates showed resistance toward one further class of antimicrobials, 24 toward two additional classes, 32 toward three additional classes and 3 toward antimicrobials belonging to four additional substance classes (**Table 1**).

Unfortunately, three isolates were, by mistake, not stored and were therefore not included in the further WGS-based analysis process. Genes conferring β-lactam resistance and other antimicrobial resistances (**Supplementary Table 1**) identified in the remaining 82 whole genome sequences were in concordance with the AST profiles (**Supplementary Table 1**). The most predominate beta-lactam resistance gene identified in this study was $bla_{CTX-M-1}$ ($n = 49$), followed by $bla_{CTX-M-15}$ ($n = 13$), bla_{SHV-12} ($n = 11$), bla_{OXA-1} ($n = 7$) and $bla_{CTX-M-14}$ ($n = 5$). One isolate carried $bla_{CTX-M-3}$ ($n = 1$) and the broadened β-lactam resistance was conferred by pAmpC (bla_{CMY-2}) in one case (**Figure 3** and **Supplementary Table 1**). Aminoglycoside resistance genes were most frequently associated with ESBL-EC (74/82 genomes, 90%), especially *aac* (3) variants (67 genomes, 82%), followed by genes enhancing resistance toward sulfonamides (*sul*1 and/or *sul*2, 89%) and trimethoprim

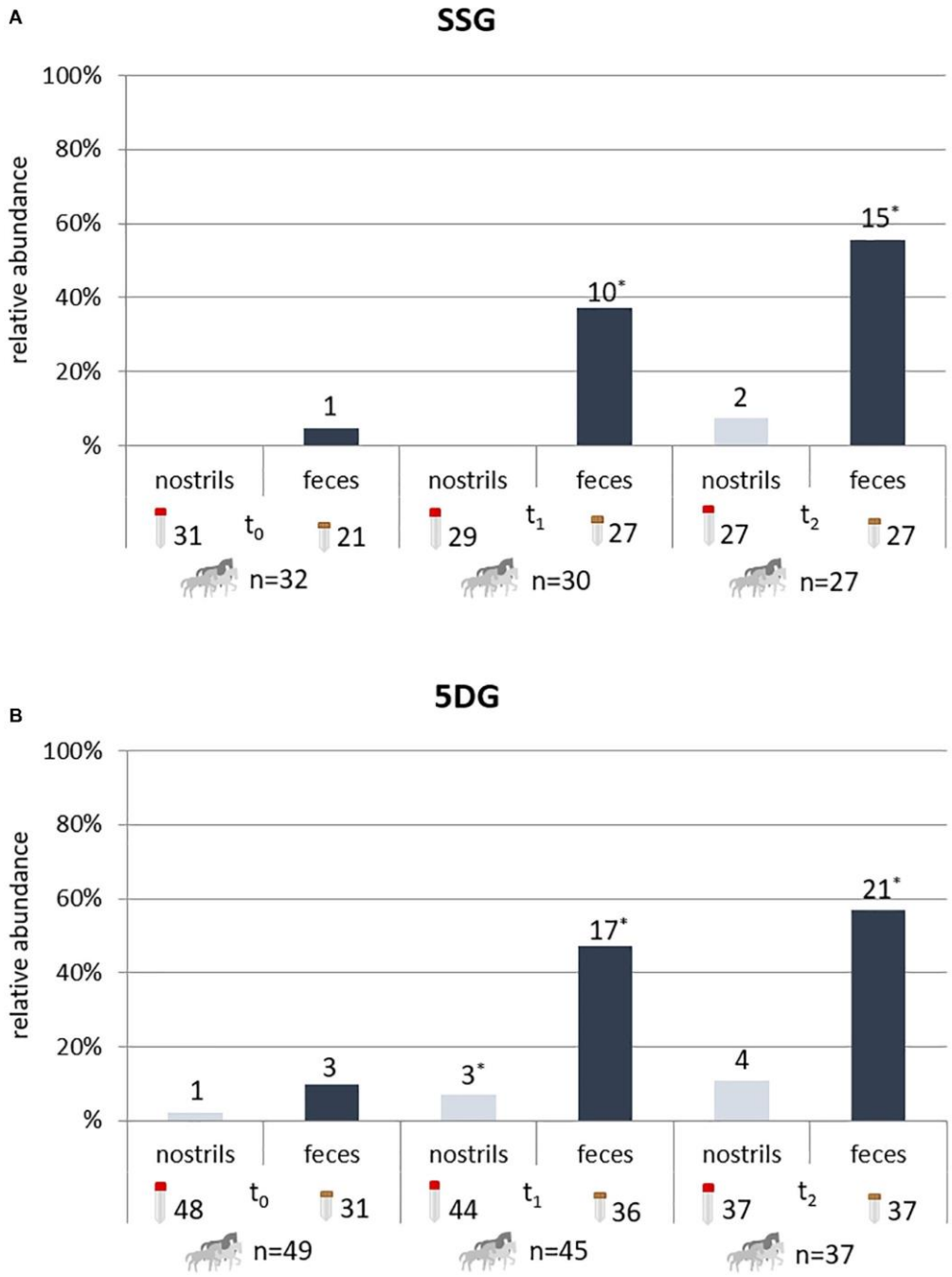

FIGURE 2 | Horses with ESBL-EC-positive specimens during hospital stay. Horses with ESBL-EC-positive specimens (nostril swabs and fecal samples) at t_0 (hospital admission), t_1 (3 days after surgery) and t_2 (10 days after surgery). Since defecation disorders are frequently associated with colic syndrome, fecal samples were not available for all horses at every time point. For the single-shot group (SSG), 32 horses met the criteria for participation in this study at t_0 **(A)**, with 31 nostril swabs and 21 fecal available for the screening procedure. Of the 49 horses initially allotted to the 5-day group (5DG), 48 nostril swabs and 31 fecal samples were collected at t_0 **(B)**. Due to the strict study design, specimens of horses failing the inclusion criteria in the ongoing study were excluded from further consideration at t_1 and/or t_2, resulting in 27 participating horses for the SSG and 37 for the 5DG at t_2. *, more than one isolate/specimen possible.

TABLE 1 | AST profiles of ESBL-EC isolated from specimens of hospitalized horses.

ST	n	ENR	GEN	SXT	TET
10	12	≤0.12	**≥16**	**≥320**	**≥16**
	1	≤ 0.12	≤1	≤20	≤1
	2	**1**	**≥16**	**≥320**	≤1
	1	**0.5**	**≥16**	**≥320**	**≥16**
	1	**0.5**	**≥16**	**≥320**	≤1
	2	**≥4**	**≥16**	**≥320**	**≥16**
1,245	5	≤0.12	**≥16**	**≥320**	=1
1,250	2	≤0.12	**≥16**	**≥320**	≤1
	1	≤0.12	**≥16**	**≥320**	=16
	1	**0.5**	**≥16**	**≥320**	≤1
	1	≤0.12	≤1	**≥320**	≤1
224	5	**≥4**	**≥16**	**≥320**	**≥16**
410	3	**≥4**	**8**	**≥320**	=16
	4	**≥4**	**≥16**	**≥320**	**≥16**
	2	**0.5**	**8**	**≥320**	**≥16**
	1	≤0.12	≤1	≤20	≤1
641	1	≤0.12	**≥16**	**≥320**	≤1
	6	≤0,12	**≥16**	**≥320**	**≥16**
	1	**0.5**	**≥16**	**≥320**	≤1
	1	**≥4**	**≥16**	**≥320**	**≥16**
86	2	**1**	**≥16**	**≥320**	**≥16**
	6	**1**	**≥16**	**≥320**	**≥16**
155	2	≤0.12	**≥16**	**≥320**	≤1
1,686	1	≤0.12	**≥16**	**≥320**	**≥16**
	1	**1**	=16	**≥320**	≤1
1,730	1	**0.5**	≤1	≤20	≤1
	1	**1**	≤1	≤20	≤1
2,035	1	**1**	**≥16**	**≥320**	≤1
2,325	1	**1**	≤1	≤20	≤1
2,350	1	≤0.12	**≥16**	≤20	≤1
	1	**0.5**	**≥16**	**≥320**	**≥16**
617	3	**≥4**	**≥16**	**≥320**	**≥16**
648	1	**≥4**	**≥16**	**≥320**	**≥16**
6,589	1	**0.5**	**≥16**	**≥320**	≤1
7,459	1	≤0.12	≤1	≤20	≤1
453	1	0.25	≤1	≤20	≤1
973	1	≤0.12	≤1	≤20	≤1
NA	1	**1**	≤1	**≥320**	**≥16**
Novel ST	1	**1**	**≥16**	**≥320**	**≥16**
NA	1	≤0.12	**≥16**	**≥320**	**≥16**
1,204	1	**1**	**≥16**	**≥320**	≤1
NA	1	**0.5**	**≥16**	**≥320**	≤1
1,709	1	≤0.12	**≥16**	**≥320**	=1

Antimicrobial susceptibility testing (AST) results of ESBL-producing E. coli. In order to assess the importance of the AST profiles with respect to therapy options in case of infection, plain numbers indicate susceptibility and bold ones a resistant phenotype.

ST, sequence type; n, number of isolates; ENR, enrofloxacin; GEN, gentamicin; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline.

(*dfr*A and *dfr*G genes, 88%). More details on the distribution of antimicrobial resistance genes in ESBL-EC isolates are provided in **Supplementary Table 1**.

Phylogenetic Background of ESBL/pAmpC-EC Isolated From Hospitalized Horses

A phylogenetic tree was generated from 2,700 orthologs genes identified using WGS data (**Figure 3**). Based on WGS analysis, 21 different STs were identified (**Supplementary Table 1** and **Figure 3**), including STs belonging to three dominating STC, i.e., STC10 (ST10; 23%), STC23 (ST410, 12%), and STC86 including ST86 (10%), ST641 (11%), and ST453 (1.2%), revealing an overall broad heterogeneity of phylogenetic backgrounds for the ESBL/pAmpC-EC isolated in this study.

STs associated with isolates from only one study group occurred rarely (e.g., ST453, n = 1, 5DG; ST617, n = 2, 5DG; ST2035, n = 2, SSG; ST1709, n = 1, SSG), a detailed illustration on the distribution of STs among the study groups is provided in **Figure 4**.

Considering all ESBL-EC-positive nostril samples (n = 12; 5.5%), only one horse (54, **Figure 3**) was positive for ESBL-EC ST10 $bla_{CTX-M-1}$ twice (t_1 and t_2). The frequency of ESBL-EC isolated from nostril swabs increased during hospitalization (t0 = 1; t1 = 4, t2 = 7: comment: some swabs were positive for more than one phenotype). The nostril swabs and fecal samples of the horses with ID41 (t_2, ST10 $bla_{CTX-M-1}$) and ID69 (t_2, ST410 $bla_{CTX-M-15}$) were positive for the same ESBL-EC clone (**Figure 5**). Contrary, the clonal background of the nostril/fecal isolates of horses with the IDs 20 (t_2: ST10 $bla_{CTX-M-1}$/ST1245 $bla_{CTX-M-14}$), 49 (t2: ST10 $bla_{CTX-M-1}$/ST155 $bla_{CTX-M-1}$), and 78 (t2: ST-224 $bla_{CTX-M-1}$/ST641 $bla_{CTX-M-1}$) were different (**Supplementary Table 1**).

Overall, fecal samples collected from 18 horses (n = 7 from the SSG and n = 11 from the 5DG) were ESBL-EC positive at two different time-points (t_1 and t_2). In two cases, isolates from t_1 and t_2 differed by two SNPs (5DG, ID26, ST617 $bla_{CTX-M-15}$ and 5DG, ID58, ST86 bla_{SHV-12}) and were therefore considered as the same clone, respectively.

While all ST86 ESBL-EC were associated with bla_{SHV-12}, distantly related ST1730 isolates were positive for bla_{SHV-12} as well. Further associations were obvious for $bla_{CTX-M-14}$ with ST1245, while $bla_{CTX-M-15}$ was identified in various clonal backgrounds (ST617, ST973, ST410, ST648, and ST2325) (**Supplementary Table 1**). Likewise, $bla_{CTX-M-1}$ was distributed across multiple genomic lineages (**Supplementary Table 1**). *In silico* detection of plasmid replicons revealed variation for the number of replicons associated with the isolates as well as a broad range of different replicon types, including IncFIA/B and IncFII (**Supplementary Table 1**).

Spatio-Temporal Dynamics of ESBL-EC

Since we noticed an increase of ESBL-EC isolation rates in specimens of horses belonging to both study groups during hospital stay, questions concerning spatio-temporal dynamics and persistence of distinct genomic lineages within the clinic (**Figures 3**, **4**) have been raised. A scatter plot displaying SNP distances, calculated for the ESBL-EC genomes of those isolates belonging to the predominating STCs, was used to further investigate the suspected local spreads.

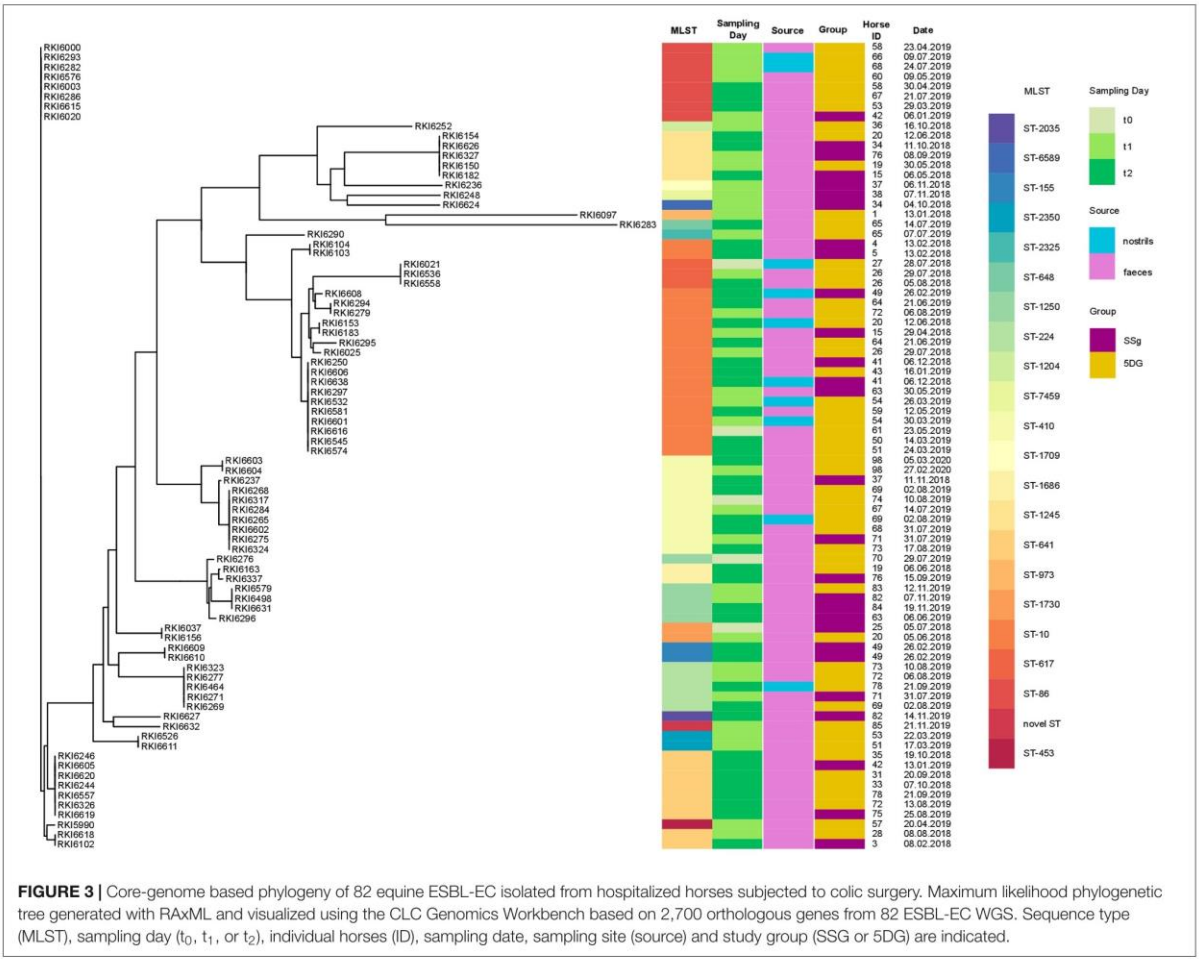

FIGURE 3 | Core-genome based phylogeny of 82 equine ESBL-EC isolated from hospitalized horses subjected to colic surgery. Maximum likelihood phylogenetic tree generated with RAxML and visualized using the CLC Genomics Workbench based on 2,700 orthologous genes from 82 ESBL-EC WGS. Sequence type (MLST), sampling day (t_0, t_1, or t_2), individual horses (ID), sampling date, sampling site (source) and study group (SSG or 5DG) are indicated.

The illustration shows the time of hospital admission and the study group assignment for individual horses. ESBL-EC-positive samples at t_0, t_1, and t_2 and close phylogenetic relationships with isolates obtained from specimens of other horses (**Figures 5A–C**) are indicated. The fecal sample of horse 5, for instance, was positive for an ST10 ESBL-EC at t_2 with no recent history (i.e., t_0, t_1) of ESBL-positive specimens in our study (**Figure 5A**). In addition, an indistinguishable clone was identified in the t_2-sample of horse 4, a horse which also had no ESBL-positive specimens before (**Supplementary Table 1**). Since the next ST10 ESBL-EC genome included here differed by at least 5,982 SNPs (**Supplementary Figure 1**), a common source of both isolates such as other (hospitalized) horses and/or the hospital environment seems likely, especially considering their close temporal relationship (**Figure 5A**).

Several additional close spatio-temporal relationships of ST10 ESBL-EC genomes are shown in **Figure 5A**. While there were "genomic singletons," e.g., from samples of the horses 26 and 49, many further genomes were classified as closely related (**Supplementary Figures 1–3**), including indistinguishable genomes for isolates of the equine patients 41 and 43 (0 SNP difference); horse 54 and 59 (1 SNP difference) as well as horse 59 and 63 (2 SNP difference) and horse 64 and 72 (2 SNP difference). Considering isolation dates for isolates belonging to STC86, a direct or indirect link most likely exists between ST641 ESBL-EC obtained from fecal samples of horse 31 (t_1) and 35 (t_2). Moreover, a total of 7 closely related genomes (0–3 SNP difference) have been identified for ESBL-EC from specimens of horses hospitalized between 9/2018 and 9/2019 (**Figure 5B** and **Supplementary Figure 2**). A further cluster comprised 9 ST86 ESBL-EC from 8 horses, including 5 indistinguishable (0 SNP difference) genomes (**Figure 5B**). It should be noted that none of these horses were associated with an STC86 ESBL-EC specimen at hospital admission (**Figure 5B**). A cluster of 8 closely related (0–2 SNP difference) ST410 ESBL-EC genomes (STC23) was identified for specimens from 6 horses hospitalized in July and August 2019 (**Figure 5C**), once more strongly indicating a local and temporary spread and transmission of this particular clone as well.

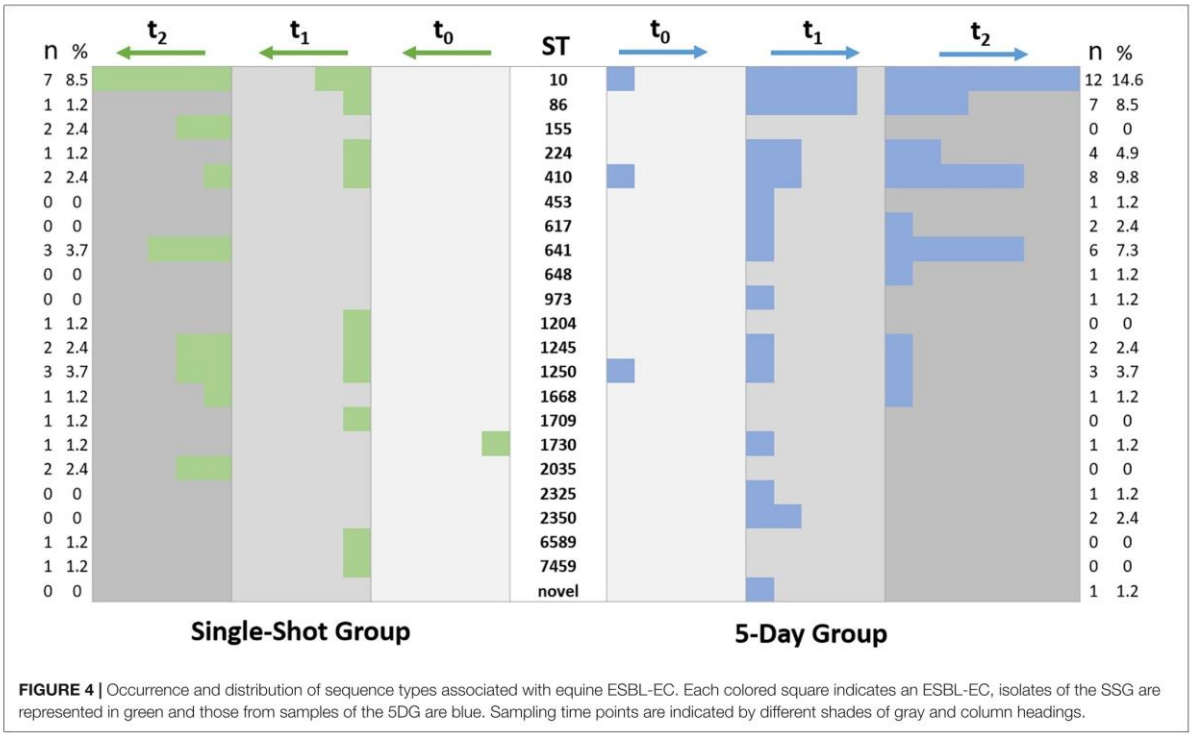

FIGURE 4 | Occurrence and distribution of sequence types associated with equine ESBL-EC. Each colored square indicates an ESBL-EC, isolates of the SSG are represented in green and those from samples of the 5DG are blue. Sampling time points are indicated by different shades of gray and column headings.

DISCUSSION

This study showed that the proportion of fecal samples positive for ESBL-EC increases among hospitalized horses subjected to median laparotomy. It further provided evidence for the local spread of distinct MDR ESBL-EC clones and the local predominance of isolates belonging to ST10, STC86 and ST641 backgrounds. While the original intention of the present study was to comparatively investigate the particular influence of a short versus a prolonged P/G PAP regime on ESBL-EC isolation rates, the overwhelming effect of hospitalization seems to outmatch the (putative) differences between distinct P/G PAP courses.

Isolation Rates of ESBL- EC in Specimens of Equine Patients

Caution is always needed when comparing study results with respect to study designs, population characteristics, local circumstances and methodical protocols used for ESBL (and pAmpC)-EC detection. In the present study, the observed ESBL-EC rates for fecal samples collected at hospital admission ranged from 5% (SSG) to 10% (5DG). Detected rates are in general concordance with previous studies, reporting proportions from 7 to 16% (Maddox et al., 2012; Walther et al., 2018b; Schoster et al., 2020), but lower than in studies using selective enrichment (Shnaiderman-Torban et al., 2020). A very recent study by Schoster et al. (2020) on hospitalized horses shedding ESBL-EC included different groups of equine patients while the duration of the PAP or anti-infective antimicrobial courses was determined individually for each patient by the veterinarian in charge. The fecal samples were collected -amongst other times- at hospital admission and 48–60 h afterward (Schoster et al., 2020). Interestingly, Schoster et al. (2020) found no significant difference concerning the ESBL-EC shedding rates in horses receiving either a fourth-generation cephalosporin or the common P/G combination. Although neither the study design nor the study population included by that particular work are directly comparable with the present study, interesting similarities are apparent: An increase in ESBL-EC detection rates reported for samples of hospitalized horses, for instance, are clearly in line with those recorded in the present study, since they have also noted an elevation in detection rates beyond 50% within 3 days after hospital admission. Schoster et al. (2020) also reported that the ESBL-EC isolation rates increased from 6.9 to 36% within 3 days after admission in horses which received no antibiotics at all. Consequently, a direct and overwhelming influence of hospitalization on the actual detection rate for samples from equine patients seems reasonable, probably also masking the effects of different P/G PAP regimes in the present study. However, since the ESBL-EC detection was lower in fecal samples obtained from the SSG, especially at t_1 (**Figure 2**), a beneficial effect of less selective pressure associated with the short-term P/G PAP regime seems likely. Nonetheless, due to the limited number of study participants, the difference lacked statistical significance.

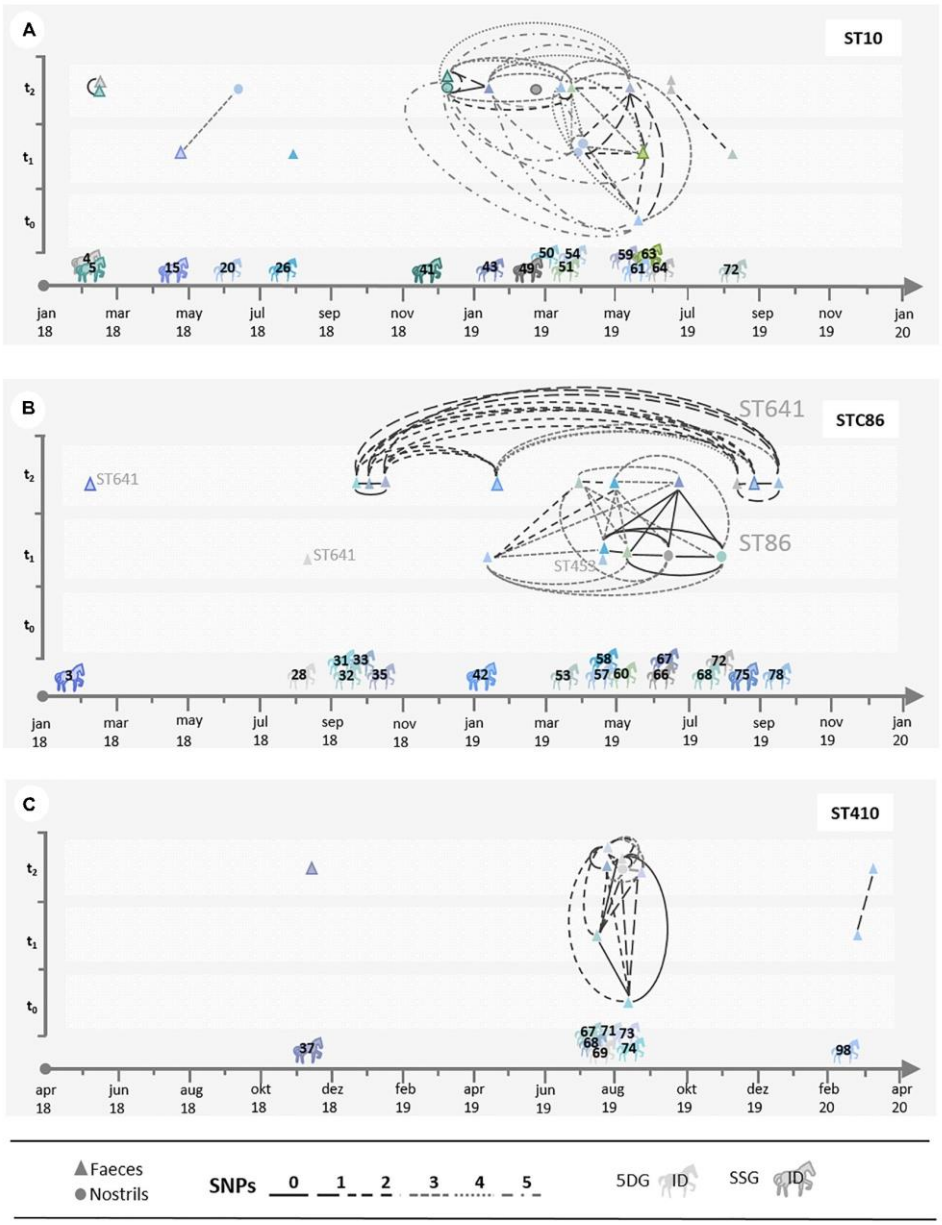

FIGURE 5 | Local spread of distinct ESBL-EC. The illustrations **(A–C)** show scatter plots displaying SNP distances calculated for the most prevalent ESBL-EC lineages on a time scale. In order to assess the possible phylogenetic and temporal relationships between the isolates, isolates of each horse (indicated by its identification number (ID)) share a particular color. Isolates gained from nostril swabs are indicated by circles, fecal isolates by triangles. The number of SNPs between two isolates is marked with a specific line. **(A)** Shows all isolates belonging to ST10; **(B)** STC86 (ST641 and ST86); and **(C)** ST410.

A recent study on non-hospitalized horses identified antibiotic treatment and veterinary examinations as risk factors associated with ESBL-EC carriage (Kaspar et al., 2019). Further studies on the subject are clearly needed, especially since antimicrobial consumption was reported being among the most important risk factors for ESBL-EC colonization in human medicine (Harris et al., 2007).

One might argue that, if a single location is not sufficient to provide robust numbers of study participants, including additional clinics might enhance the study power. On the

other hand, as recently shown by various authors, the local MDR burden tends to differ, especially during hospitalization (Apostolakos et al., 2017; Isgren et al., 2019; Hordijk et al., 2020; Shnaiderman-Torban et al., 2020), indicating a potential source of bias when considering multicenter studies. In addition, it seems likely that technical, structural and hygiene circumstances differ between facilities providing health care for horses, since overall comparability of standards, to the best of the authors' knowledge, has not been achieved yet.

Antibiotic Susceptibility Profiles and Antimicrobial Resistance Genes

In this study, the predominating ESBL-genes were $bla_{CTX-M-1}$ (59.7%) and $bla_{CTX-M-15}$ (15.8%), which supports earlier findings (Lupo et al., 2018; Walther et al., 2018b; Isgren et al., 2019; Hordijk et al., 2020).

Most of the isolates investigated in this study showed not only broadened resistance associated with their β-lactam resistance but also reduced susceptibility to other antimicrobial substances as well: Most of the ESBL-EC (51/85; 60%) isolates fulfilled the criteria for MDR (Schwarz et al., 2010). These MDR ESBL-EC pose therapeutic challenges for veterinarians since resistance genes conferring resistance to aminoglycosides, tetracyclines and trimethoprim/sulfamethoxazole are often present on the same plasmid (Li et al., 2019). Since the panel of antimicrobial substances currently available for approved administration to horses is very limited and includes the above mentioned antimicrobials (Maddox et al., 2012; Walther et al., 2014a), targeted strategies and guidelines are needed to cope with the apparent clinical challenges in equine antimicrobial therapy, especially to prevent hospital-associated infections and to reduce potential transmission between different sources and hosts, including humans (Walther et al., 2014b; Apostolakos et al., 2017; Royden et al., 2019; Hordijk et al., 2020).

Phylogenetic Background of ESBL-EC Isolated From Samples of Hospitalized Horses

In total, 21 different STs have been identified for the ESBL-EC and the single pAmpC-EC, confirming earlier studies which reported a broad heterogeneity of phylogenetic backgrounds for ESBL-EC in horses (Apostolakos et al., 2017; Walther et al., 2018b; Schoster et al., 2020). Different ESBL-EC have been detected in a single sample, e.g., ESBL-EC belonging to ST10 and ST1245 in the "t_2 fecal sample" of horse 20 (**Supplementary Table 1**). Hence, including different phenotypes in the ESBL-EC screening process is mandatory, as previously reported by Apostolakos et al. (2017). Considering the occurrence and broad distribution of STs among the isolate collection investigated (**Figure 4**), no obvious differences were identified.

However, ST10, STC86 (ST86, ST641, and ST453) and ST410 were the predominating phylogenetic lineages associated with the isolate collection, and assessment of their genomic relatedness revealed a close relationship (based on SNP differences) between many of the isolates belonging to a particular ST. Of note, neither ST86 nor ST641 were found to be associated with ESCL-EC isolated from specimens taken at hospital admission (**Figure 5B**).

Consequently, a common source and/or direct or indirect transmission of these ESBL-EC (**Figure 3**) seems more likely than spread of certain plasmids carrying β-lactam resistance genes, as previously suggested (Walther et al., 2018b). This hypothesis is supported by the finding that the overall diversity of genetic backgrounds seems to have decreased over time: while 18 different STs were associated with the 35 isolates obtained at t_1, the 45 isolates representing t_2 belonged to only 12 different STs, with ST10 (12 isolates), ST641 (8 isolates), and ST410 (6 isolates) being dominating lineages. Moreover, previous reports from Germany, the Netherlands and the United Kingdom also described ESBL-EC belonging to ST10, STC86, and ST410 for samples of horse origin as among the predominating lineages (Apostolakos et al., 2017; Walther et al., 2018b; Bortolami et al., 2019; Schoster et al., 2020).

Of note, MDR ESBL-EC belonging to ST10 were only recently reported for samples of meat, poultry and wildlife origin as well as for clinical human samples in Spain, once more emphasizing that this lineage is able to adapt to different life circumstances while crossing the borders of different niches and hosts (Diaz-Jimenez et al., 2020). Similar to the ubiquitous occurrence of *E. coli*-ST10, *E. coli* belonging to ST410 have also been described for samples from various hosts, including companion animals, livestock animals, wildlife- and human samples (Falgenhauer et al., 2016; Fischer et al., 2017; Reynolds et al., 2019). Moreover, ST410 was previously described as a genomic lineage containing high-risk trans-sectoral transmissible and multidrug-resistant clones in veterinary as well as human medicine (Schaufler et al., 2016; Roer et al., 2018). A recent study by Touchon et al. (2020) found that phylogeny and habitat shape the genetic diversification of *E. coli* to similar extents, and diversification might occur by acquiring genes and mobile elements not only from other gut-residing bacteria but from (well-established) environmental bacteria as well.

Spatio-Temporal Dynamics of ESBL-EC During Hospitalization

Considering the nostril swabs that were occasionally found positive for ESBL-EC, we detected the same clone (0–2 SNPs difference) in fecal samples as well, e.g., for horse 69 (t_2; ST-410 *bla*CTX-M-15) or horse 41 (t_2, ST10 *bla*CTX-M-1). On the other hand, we also identified a single clone (ST86 bla_{SHV-12}) in nostril swabs of two distinct horses (t_1, horse 66 and 68), (for details see **Supplementary Table 1** and **Figures 5A–C**). Previous studies which included environmental sampling identified ESBL-EC contamination of horse stables, the floor, medical equipment and other sites as a possible source for in-ho transmission (Walther et al., 2014b; Schoster et al., 2020). While the detection of ESBL-EC in nostril swabs screened at hospital admission was assumed to represent prior contamination, for instance associated with nasogastric intubation (Walther et al., 2018b), sampling results presented here point toward a direct or indirect transmission during hospitalization. These results are in concordance with previous research revealing veterinary

clinics as "hotspots" for transmission of MDR Enterobacterales (Wright et al., 2005; Apostolakos et al., 2017; Walther et al., 2018b). Some closely related isolates were able to spread over several months-for example, in the period from January to August 2019 (STC10 and STC86). Last but not least, contact with horses was previously identified as a risk factor for humans to become colonized by ESBL-producing bacteria (Huijbers et al., 2013). Since our results are in line with recent reports from other horse clinics, we are confident that local accumulation and spread together with the presence of vulnerable patients receiving antibiotics and other selective agents are the main drivers for the presented observations. Therefore, increasing awareness for the importance of hygiene is necessary to cope with the current challenges presented by MDR and zoonotic pathogens in horse clinics: As a direct consequence of our study results, hygiene recommendations for horse clinics have been recently published (Gehlen et al., 2020).

CONCLUSION

MDR pathogen accumulation in horse clinics, including ESBL-EC, is a threat to both, the equine patients and the people working with and around them. Considering the proverb "an ounce of prevention is worth a pound of cure," the time to critically re-consider hygiene recommendations and common protocols for the administration of antibiotics in equine medicine appears overdue, especially if prolonged antibiotic courses seem to lack scientific proof of superior outcomes in patients, which needs to be addressed thoroughly in further studies.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

According to the German regulation authorities for research with animal subjects, the comparison of two perioperative prophylaxis regimens in horses subjected to colic surgery does not require approval (Landesamt für Gesundheit und Soziales, Berlin, 18.04.2017). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

BW, AL-B, and HG designed the project. SS, HG, and BW conceived and designed the experiments. TS sequenced the isolates. AK, DK, SS, HG, and AL-B performed laboratory analysis. BW, LE, AL-B, SW, TS, SG, and FG analyzed the data. AK, SW, and BW wrote the article. All authors have read and approved the final draft of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.671676/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

4.3. **Veröffentlichung III**

Antibiotic prophylaxis and hospitalization of horses subjected to median laparotomy: gut microbiota trajectories and abundance increase of *Escherichia*

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Beiträge der Autoren

BW, AL-B, LHW und HG entwarfen das Projekt. AK, SDS, HG und BW planten und gestalteten die Experimente. AK, DK, SDS, JB und AL-B führten die Laboranalysen durch. CB und SF sequenzierten die Proben. AK, SAW, AL-B, BW und TS analysierten die Daten. AK, SAW und BW verfassten den ersten Entwurf. AM, NE, RK und SG halfen bei der Erstellung des Manuskripts und trugen zur Diskussion bei.

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Antibiotic prophylaxis and hospitalization of horses subjected to median laparotomy: gut microbiota trajectories and abundance increase of *Escherichia*

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Abstract

Horse clinics are hotspots for the accumulation and spread of clinically relevant and zoonotic multidrug-resistant bacteria, including extended-spectrum β-lactamase producing (ESBL) Enterobacterales. Although median laparotomy in cases of acute equine colic is a frequently performed surgical intervention, knowledge about the effects of peri-operative antibiotic prophylaxis (PAP) based on a combination of penicillin and gentamicin on the gut microbiota is limited. Therefore, we collected fecal samples of horses from a non-hospitalized control group (CG) and from horses receiving either a pre-surgical single-shot (SSG) or a peri-operative 5-day (5DG) course of PAP. To assess differences between the two PAP regimens and the CG, all samples obtained at hospital admission (t_0), on days three (t_1) and ten (t_2) after surgery, were screened for ESBL-producing Enterobacterales and subjected to 16S rRNA V1–V2 gene sequencing.

We included 48 samples in the SSG (n=16 horses), 45 in the 5DG (n=15) and 20 in the CG (n=10). Two samples (6.5%) were positive for ESBL-producing Enterobacterales at t_0 while this rate increased to 67% at t_1 and decreased only slightly at t_2 (61%). Shannon diversity index (SDI) was used to evaluate alpha-diversity changes, revealing that horses suffering from acute colic seemed to have a compromised fecal microbiota composition (5DG, SDI_{mean} of 5.90; SSG, SDI_{mean} of 6.17) when compared to the CG (SDI_{mean} of 6.53) at t_0, although the difference lacked significance. Alpha-diversity decreased significantly in both PAP groups at t_1, while at t_2 the onset of microbiome recovery was noticed. Although we did not identify a significant SDI_{mean} difference with respect to PAP duration, the community structure (beta-diversity) was considerably restricted in samples of the 5DG at t_1, most likely due to the ongoing administration of antibiotics. An increased abundance of *Enterobacteriaceae,* especially *Escherichia*, was noted for both study groups at t_1. Further studies are needed to reveal important factors promoting the increase and residency of ESBL-producing Enterobacterales among hospitalized horses.

1 Introduction

Compared with other companion animals, horses more often acquire gastro-intestinal tract (GIT) disorders that may lead to long-term suffering or even death (Traub-Dargatz et al., 2001). The syndrome complex pain caused by disorders of the GIT in horses is commonly referred to as “colic” (Traub-Dargatz et al., 2001;Stockle et al., 2018). The composition of the bacterial community residing within the GIT has been regarded as beneficial and a prerequisite for the health and well-being of hindgut fermenters such as Equidaes (reviewed in (Kauter et al., 2019)). Previous reports indicated that colonization with the physiological endogenous microbiota shields the equine GIT against either direct or indirect pathogen-induced damages and that these protective effects are perturbed throughout various enteral maladies (Costa et al., 2012;Weese et al., 2015). Moreover, administration of antibiotics such as penicillin (Baverud et al., 2003), enrofloxacin or ceftiofur (Liepman et al., 2022), as well as doxycycline (Davis et al., 2006) were reported to drive the enteral microbial community towards a dysbiotic state (Costa et al., 2015). However, besides other factors, the state of the microbiota at the time of (antibiotic) perturbation (diet, species, and functional diversity and redundancy) and the characteristics of the perturbation (e.g. administration route, antimicrobial spectrum, and duration of antibiotic courses) determine the extent of any eventual dysbiosis (Schwartz et al., 2020).

To prevent adverse postoperative events such as surgical site infections in horses subjected to abdominal surgery due to acute colic, peri-operative antibiotic prophylaxis (PAP) is recommended (Dallap Schaer et al., 2012). The most commonly used PAP regimen for horses requiring median laparotomy consists of a combination of penicillin and gentamicin (P/G) for a period of 3-10 days (Dallap Schaer et al., 2012;Wormstrand et al., 2014;Teschner et al., 2015). In contrast, the standard regimen for similar surgical interventions lacking complicating circumstances in human and small animal medicine is a short-term PAP, which is provided as a single-shot therapy 30-60 minutes prior to incision (Stöckle et al., 2021). To investigate the effects of prolonged administration

beyond this immediate peri-operative timeframe (> 24 h after surgery), we conducted a pilot study focusing on the clinical outcome (Stöckle et al., 2021), the extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* (EC) carriage rates (Kauter et al., 2021) and the microbiome composition (present study). Beyond others, our results indicated non-inferiority of a "single-shot" versus a five-day course of P/G PAP with respect to the patients' clinical outcomes (Stöckle et al., 2021). Regardless of the applied PAP-regimen, we noticed a worrisome increase of ESBL-EC carriage rates among these horses during their hospital stay (Kauter et al., 2021).

Based on these previous observations, the current study aimed i) to examine the extent of microbiome disturbance in hospitalized horses subjected to median laparotomy, ii) to reveal alterations of the gut microbiota caused by a short and a long-term PAP regime and iii) to enable insights into changes of the microbiome that might play a role in the previously reported increased ESBL-EC carriage rates among equine patients.

2 Materials and Methods

Study cohort and perioperative antibiotic prophylaxis

Horses diagnosed with acute abdominal pain (colic syndrome complex) that required median laparotomy were included in this study (detailed description of the controlled and randomized pilot study in (Stöckle et al., 2021)). Briefly, horses allotted to the SSG received short term P/G PAP, while the 5DG group received P/G PAP for five consecutive days. In both groups, PAP consisted of parenteral administration of sodium penicillin G (22,000 IU/kg four times daily) and gentamicin (6.6 mg/kg), as previously recommended for colic surgery (Dallap Schaer et al., 2012;Durward-Akhurst et al., 2013;Southwood, 2014). Specimens of ten non-hospitalized farm horses that were free of clinical symptoms for any apparent illnesses served as a control group (CG). The latter were sampled twice within three days to ensure representativeness of the results (detailed description of the study participants and their respective clinical

outcomes in (Stöckle et al., 2021)). A graphical abstract of the study outline is provided in **Figure 1**.

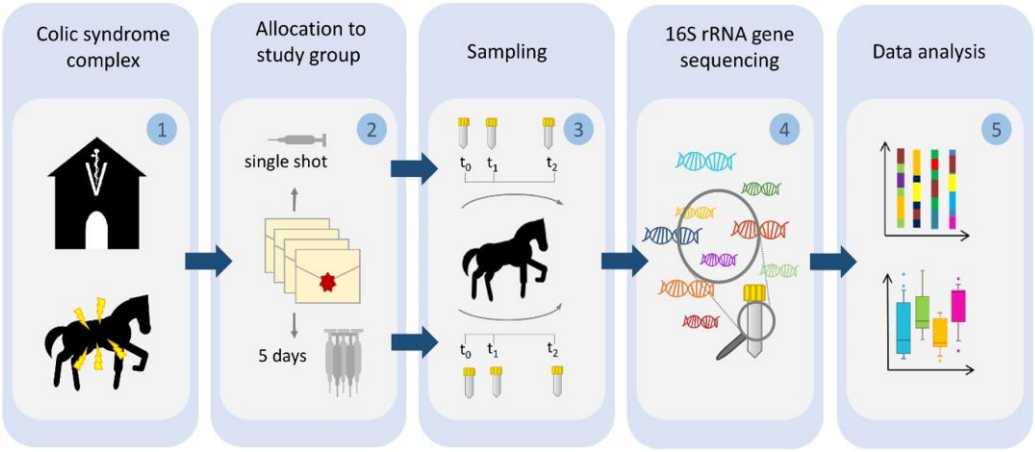

Figure 1| Illustration of the sampling procedure

Horses subjected to colic surgery were **(1)** sampled directly at hospital admission (t_0) and **(2)** allotted to either a single-shot perioperative antibiotic prophylaxis regimen or a 5-day-lasting protocol. Sampling was repeated for all horses on day 3 (t_1) and 10 (t_2) after surgery **(3)**. All samples were subjected to DNA extraction and V1–V2 16S rRNA gene sequencing **(4)**. Sequences were preprocessed and analyzed with respect to changes of microbiota composition, alpha and beta diversity **(5)**.

Inclusion criteria for study participation were: study participants had to be free of clinical signs of infectious diseases prior to surgery. Additionally, since the juvenile microbiome is known for continuing changes during the foals' gut maturation (Costa et al., 2016), all included horses were required to be one year or older. Equine patients were excluded from further consideration if their hospital stay had ended prematurely due to euthanasia and/or the antibiotic regimen they had originally been assigned to was not strictly followed, regardless of the particular reasons requiring these changes. Horses with incomplete sample sets (t_0, t_1, t_2, see sample collection) were also excluded (Stöckle et al., 2021).

Sample collection

Fresh fecal samples were collected from each horse diagnosed with colic syndrome complex directly at hospital admission (t_0), as described previously (Walther et al., 2018;Kauter et al., 2021). A second sample was collected after three days (t_1) and a third (final) sample was obtained ten days after surgery (t_2). In order to gain insights into the gut microbiome associated with non-hospitalized horses lacking clinical signs of gastro-intestinal disorders, control samples were obtained from ten horses residing in a barn. All specimens were stored directly at -80° C and shipped on dry ice.

Identification of ESBL-producing Enterobacterales was previously described (Kauter et al., 2021). Briefly, samples were cultured on Brilliance™ ESBL Agar plates (Thermo ScientificTM, Germany) overnight. Colonies showing characteristic growth signatures of ESBL-producing Enterobacterales on chromogenic screening plates were further investigated. In case of distinct phenotypic appearances of presumptive ESBL-producing colonies on the plates, all isolates were subjected to an ESBL confirmation test according to the Clinical Laboratory Standards Institute's (CLSI) recommendations (CLSI, 2020). Species confirmation was achieved by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (Bruker, Germany).

DNA extraction and sequencing of the bacterial 16S rRNA V1-V2 region

The sequencing of bacterial DNA was performed by the Institute of Clinical Molecular Biology (IKMB) at the Christian-Albrechts University of Kiel. DNA was extracted using the QIAamp Fast DNA stool mini kit (QIAGEN, Hilden, Germany) automated on the QIAcube (QIAGEN, Hilden, Germany). For this, approximately 200 mg feces were transferred to 0.70 mm Garnet Bead tubes filled with 1 ml InhibitEx buffer. Subsequently, bead beating was performed using a SpeedMill PLUS (QIAGEN, Hilden, Germany) for 45 s at 60 Hz. Samples were then heated to 95° C for 5 min and afterwards centrifuged for 1 min at 10,000 rpm. 200 µl of the resulting supernatant

were transferred to a 1.5 ml microcentrifuge tube, which was placed in the QIAcube for follow-up automated DNA isolation according to the manufacturer's protocol. DNA bound specifically to the QIAamp silica-gel membrane while contaminants passed through. PCR inhibitors were removed through means of InhibitEX (QIAGEN, Hilden, Germany), a unique adsorption resin, and an optimized buffer. DNA was diluted 1:10 prior to PCR, and 3 µl were used for further amplification. PCR-products were verified using agarose gel electrophoresis. PCR-products were then normalized using the SequalPrep Normalization Plate Kit (Thermo Fischer Scientific, Waltham, MA, USA), pooled equimolarily and sequenced on an Illumina MiSeq v3 2x300 base pair (bp) platform (Illumina Inc., San Diego, CA, USA).

The V1-V2 region of the 16S rRNA gene was subsequently sequenced (Primerpair 27F-338R, dual MIDs inducing) on a MiSeq-platform (MiSeq Reagent Kit v2) (Kozich et al., 2013). The resulting MiSeq raw fastq data was verified using an inhouse pipeline (Bcl2fastq Modul in CASAVA 1.8.2, Demultiplexing, FLASH software (v1.2) (Magoč and Salzberg, 2011), fastx toolkit und UCHIME (v6.0) (Edgar et al., 2011)). Demultiplexing was performed based on 0 mismatches within the barcode sequences.

Analysis of 16S rRNA gene sequences

Data Preprocessing

Sequence reads were preprocessed as described (Mach et al., 2020). In brief, paired-end reads were merged into continuous sequences using the "join_paired_ends.py" script of QIIME (v1.9.1) (Caporaso et al., 2010) in "fastq-join" mode (Aronesty, 2013). Defined parameters allowed a minimum overlap of 6 bp and a maximum difference within the overlap region of 8%. Reads which did not meet these criteria were removed from further analysis. Next, seqkit (v0.16.1) (Shen et al., 2016) was utilized to filter out reads which were too short (≤300bp) or too long (≥470bp). The remaining sequences were then quality filtered using the "split_libraries_fastq.py" script of QIIME (v1.9.1) [25] by applying a PHRED quality threshold of 20. Reads were hereby required to have

50% of their bases to be consecutively of high quality. A maximum of three consecutive low-quality bases were allowed before truncating a read. Reads containing any ambiguous bases (“N”) were removed from further analysis.

OTU Clustering

Reads were clustered into operational taxonomic units (OTUs) using USEARCH (v11.0.667) (Edgar, 2010) and the Greengenes database (release 2013-08, gg_13_8_otus, 99_otus) (DeSantis et al., 2006) with a 97% similarity cutoff. The ”unnoise3” algorithm of USEARCH was utilized for additional filtering of chimeric OTUs. In order to improve taxonomic annotation, Greengenes OTUs as well as unmatched representative sequences were then mapped against the RDP database (v11.5) (Cole et al., 2014) using the SequenceMatch pipeline of rdptools (v2.0.3) (Cole et al., 2014), searching for the closest neighbor (k=1) with a minimum sab score of 0.5. Counts were subsequently merged and the “filter_otus_from_otu_table.py” script of QIIME (v1.9.1) (Caporaso et al., 2010) was utilized to remove any singletons from the table (counts ≤ 3). Data were then exported using the biom (v 2.1.7) (McDonald et al., 2012) package and converted into the appropriate format for further data analysis. The resulting OTU table was then finalized by updating the available taxonomic labels with the recently released, new taxonomic names for bacterial phyla (Oren and Garrity, 2021).

Sample size calculation

Sample-size calculation was performed as recommended for microbiome studies by utilizing a permutation-based extension of the multivariate analysis of variance (PERMANOVA) on a matrix of pairwise distances (Kelly et al., 2015). Subsequent study power estimation was performed through the R package micropower (v0.4) (Kelly et al., 2015). Unfiltered OTU tables were hereby subjected to random rarefaction in order to assess key population parameters, including mean and standard deviation

per OTU based on both the presence/absence of individual taxa as well as their abundance (Weighted Jaccard Distance) for a comparison of two groups of fixed size (n=10). These parameters were then further utilized to simulate a range of distance matrices and effect sizes in order to estimate the statistical power for identifying an effect size given a specified sample size and respective OTU table. Permutation-based sample-size estimation, according to Kelly et al. (Kelly et al., 2015), revealed that a subset of samples from ten horses per study group were sufficient to identify differences in taxonomic composition of effect size 0.020 with p=0.05 and a power of 80% (90% to identify an effect size of 0.035).

Diversity estimation and OTUs assignation

The resulting OTU counts were randomly sub-sampled for each sample to a homogeneous level, defined by the counts of the lowest sample (12,178). OTU counts above 10,000 have been shown to provide adequate comparisons between differing sequencing depths for microbiome analyses (Mach et al., 2020). Rarefaction was performed by using the “rarefy_even_depth” function of phyloseq (v1.36.0) (McMurdie and Holmes, 2013) with rngseed=1 in R (v4.1.1). Rarefied data was then utilized to assess the influence of antibiosis on the microbiome diversity among the equine patients and to visualize the distribution of taxa (OTUs) across the sample set. Within-sample diversity (alpha-diversity) was assessed through Shannon diversity indices (SDI) calculated using the R package microbiome (v1.14.0) (Lahti et al., 2017)). Between-sample diversity (beta-diversity) was determined through the computation of Bray-Curtis distances by phyloseq (v1.36.0) (McMurdie and Holmes, 2013) on all OTUs of the rarefied table. The resulting diversity metrics were further visualized with ComplexHeatmap (v2.8.0) (Gu et al., 2016) and ggplot2 (v3.3.5) (Wickham et al., 2016) for subsequent comparisons between the study groups. Non-parametric Paired Wilcoxon Rank Sum tests were performed between groups of interest and results with a $p<0.05$ were labeled as being significant. The Benjamini-Hochberg procedure was

utilized for multiple-testing correction in order to limit the false discovery rate were applicable. OTUs were aggregated at selected taxonomic levels (including phlya) using the “aggregate_top_taxa” function of microbiome (v1.14.0) (Lahti et al., 2017). Differential taxa were identified between the PAP study groups (SSG / 5DG) using the raw OTU table and the microbiomeExplorer R package (Reeder et al., 2021) with proportional normalization and the DESeq2 method (Love et al., 2014). Additional correlation analyses were also performed within the microbiomeExplorer suite.

3 Results

To explore the effects of both G/P PAP regimens and hospitalization on the gut microbiota of horses subjected to median laparotomy, microbiome sequencing and analysis were performed using sample sets obtained from a pilot study comparing the clinical outcomes of 67 patients that met the study's inclusion criteria (Stöckle et al., 2021). Overall, 48 samples from 16 horses representing the SSG and 45 samples of 15 horses belonging to the 5DG were comparatively evaluated. As a non-hospitalized CG we included additional 20 samples of ten horses that lacked any apparent signs of clinical illness, resulting in 113 samples (**Supplementary Table 1)**. At hospital admission, two samples (6.5%) were positive for ESBL-producing Enterobacterales (**Supplementary Table 1**), while, regardless of the study group, the overall rate increased to 67% (t_1) and, only slightly decreased, at t_2 (61%). There was no difference in carriage of ESBL-producing Enterobacterales between the SSG and 5DG (Fisher's Exact Test, $p = 1$), while all CG samples were negative (Fisher's Exact Test, $p < 0.0001$).

In total, 4,896,645 high quality OTU counts (ranging between 12,178 – 220,454 counts per sample, median = 32,730) were obtained and assigned to 17,035 different OTUs across 330 different taxonomic entities at genus level. Further taxonomic assignment of these OTUs revealed the top ten bacterial taxa (phylum level) based on their total counts across the sample set: The phyla *Bacteroidota* (38%) (previously *Bacteroidetes*) (Oren and Garrity, 2021) and *Bacillota* (33%) (previously: *Firmicutes*) (Oren and Garrity, 2021) were predominant in the equine fecal samples at hospital admission (t_0), followed by *Verrucomicrobiota* (11%), *Pseudomonadota* (9%) (previously *Proteobacteria*) (Oren and Garrity, 2021) and *Spirochaetota* (4%) (**Figure 2**).

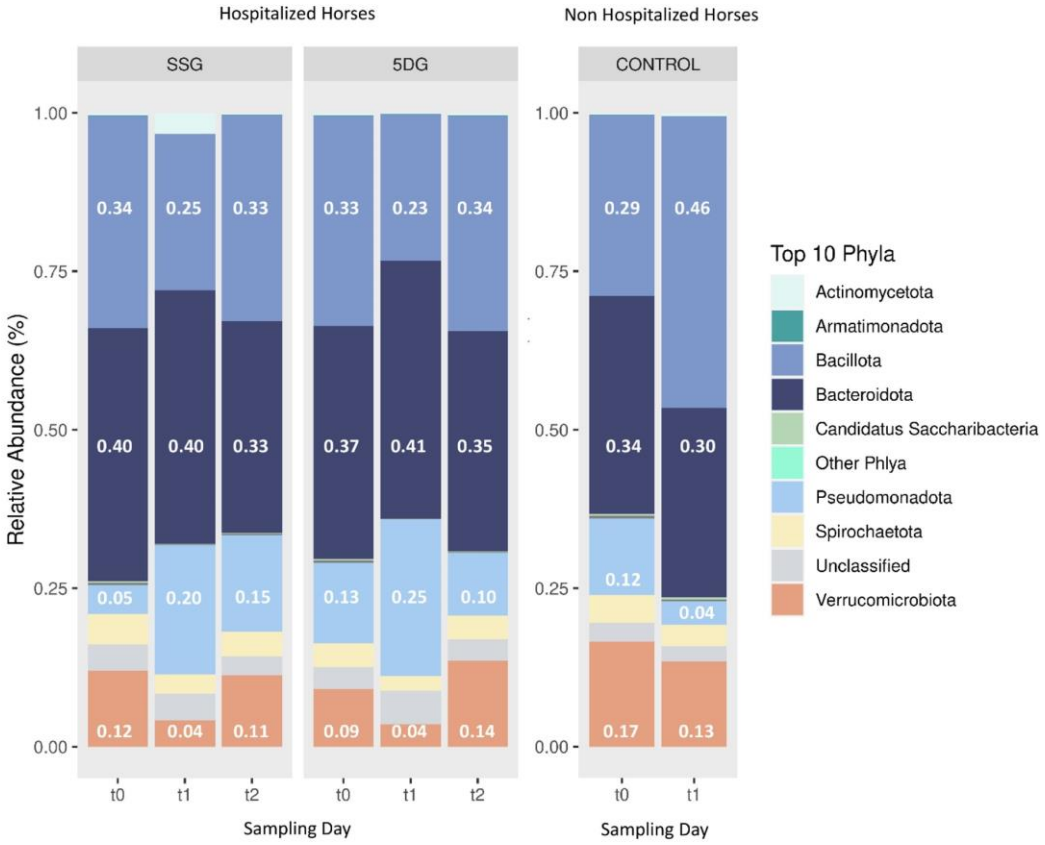

Figure 2| Taxonomic composition of microbiota in hospitalized horses

Stacked bar charts illustrating the relative abundances of the top ten phyla for the single-shot group (SSG, n=16) and the 5-day group (5DG, n=15).

Abbreviations: t_0 = hospital admission (SSG/5DG) / first sampling round (CG); t_1 = three days after surgery (SSG/5DG) / three days after first sampling (CG); t_2 = ten days after surgery (SSG/5DG).

Each horse harboured an individual composition of fecal bacterial communities, as presented in **Supplemental Figure 1**.

i) Evaluation of gut microbiota profiles, microbiome disturbance, biodiversity and microbiota trajectories

Microbiota profiles at hospital admission

The SDI is a measure of a sample's diversity based on both, community richness and -evenness. In the present study, SDI was selected to inspect the alpha-diversity within each sample and to compare these across study groups. Overall comparableness of the treatment groups was ensured by demonstrating the lack of significant difference in the mean SDI between both study groups at t_0 (Wilcoxon test, $P > 0.05$). As expected, visualization of beta-diversity ("between sample diversity") using ordination plots generated through principle component analysis (PCA) demonstrated an increased range of variance regarding the gut microbiota profiles of individual horses belonging to the SSG and the 5DG compared to the overall variance noticeable between the data points representing the CG samples (**Figure 3A-C**).

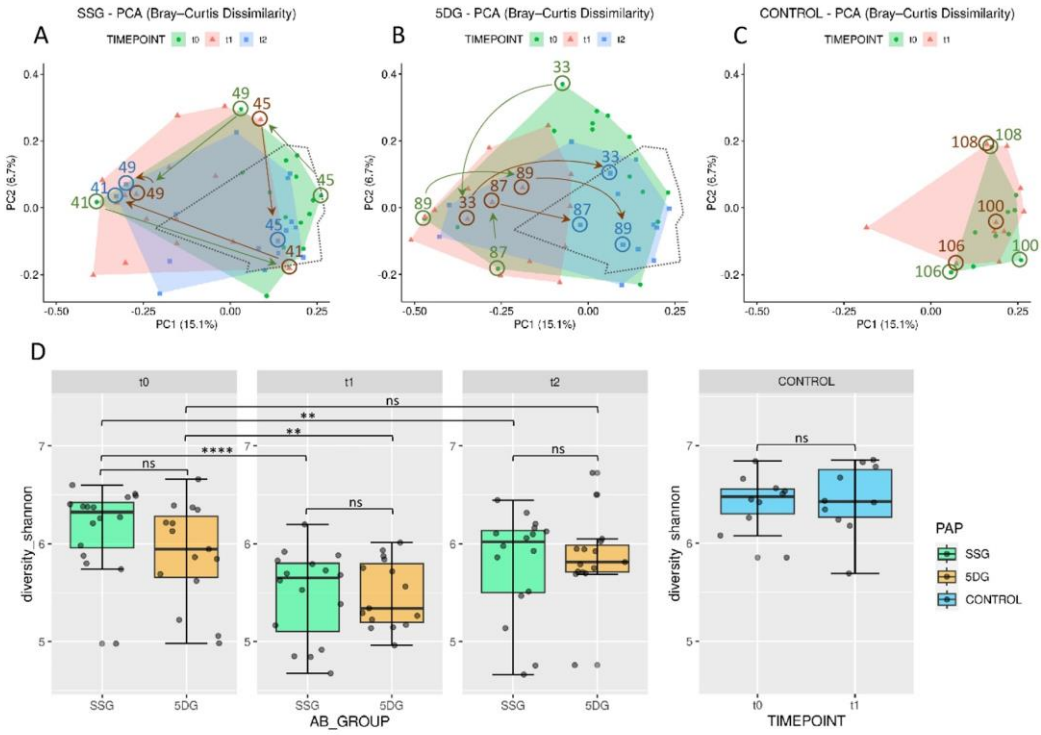

Figure 3| Diversity of the equine gut microbiome of hospitalized and non-hospitalized horses

A-C) PCA plots illustrating differences in gut microbiome composition (beta-diversity) based on unweighted Bray-Curtis dissimilarities across each group per sampling day. Axes represent the two dimensions that explain the largest proportion of variation across communities of each analysis. Distances between two data points reflect their similarity/dissimilarity with respect to sample composition. The colored areas represent the computed convex hull of data points from each sampling day, illustrating respective areas of minimal size. The area encompassing data points from the control group (CG) samples **(C)** is marked by a dotted line in **(A)** and **(B)** for direct comparison. Horses treated with peri-operative antibiotic prophylaxis (PAP) in the single-shot group (SSG) vs. the 5-day course group (5DG) show both large, intraindividual differences at t_0 **(A)** and a strong shift away from the area defined by the CG samples at t_1 **(B)**. Samples of both study groups converge back to the CG area at t2. Numbered arrows indicate the trajectories of microbiota composition within selected horses. **(D)** Boxplots displaying the alpha-diversity indices (Shannon) for all three groups (SSG, n=16; 5DG, n=15; control, n=10) (significance between groups: unpaired Wilcoxon Rank Sum tests, ** $p<0.05$, **** $p<0.0005$, ns=not significant).

Of note, the mean SDI values of the CG samples were 6.42 and 6.44, respectively. At t_0, most of the enteral microbiota profiles associated with SSG horses (11 of 16) clustered within the framework set by the CG samples (**Figure 3A**). The t_0 microbiota profiles obtained for the 5DG, on the other hand, illustrated increased variance among the 5DG samples, and only four out of 15 samples clustered with the CG area, potentially indicating that more horses belonging to the 5DG had, in comparison to the SSG, gut microbiota perturbances at hospital admission (**Figure 3B**). This observation is supported by our examination of the alpha-diversities, since the t_0 samples representing the SSG were associated with an SDI_{mean} of 6.17, while the 5DG yielded an SDI_{mean} of 5.90, although the difference lacked statistical significance (**Figure 3D**).

Microbiota profiles at t_1

At t_1, most of the microbiota profiles obtained from fecal samples of the SSG (horses without antibiotics for at least 36h) and the 5DG (horses at the third day of their 5-day course P/G PAP) visibly differed from those obtained at t_0 by shifting away from the centre of the CG area, indicating perturbations (**Figure 3B**). This shift was accompanied by a significant reduction in mean alpha-diversity across both groups [SSG: SDI from t_0 = 6.17 to t_1 = 5.48 (paired Wilcoxon Test, p=0.000031); 5DG: SDI from t_0 = 5.90 to t_1 = 5.48 (paired Wilcoxon Test, p=0.012)] (**Supplemental Table 1**, **Figure 3D**). In addition, the overall distances between 5DG samples seemed considerably restricted compared to the situation at t_0 (**Figure 3B**). Samples representing the SSG, on the other hand, were more widely scattered than samples representing the 5DG or even the CG, indicating an increased level of inter-individual differences among microbiome compositions for SSG horses at t_1.

Microbiota profiles at t_2

At t_2, i.e. ten (SSG) and five days (5DG) after the final PAP course was administered, the mean alpha-diversity of both study groups increased (SSG, SDI_{mean} = 5.80; 5DG, SDI_{mean} = 5.87), indicating the onset of microbiome recovery (**Supplemental Table 1**, **Figure 3C)**.

Common trajectories and inter-individual differences in microbiota profiles

To likewise illustrate common spatial shifts and individual deviation from common trajectories, data points representing samples of three individuals per group are highlighted in **Figure 3**:

5DG: At t_0, the data points belonging to samples of equine patients 89, 33 and 87 clustered most distantly from the CG samples, indicating a considerable disturbance of the microbiome structure and composition at hospital admission. This finding is supported by the individual samples' low SDI values (5.05, 5.75 and 5.29) (**Supplemental Table 1**). At t_1, samples 33 and 87 showed a different composition compared to the t_0 situation, but both points still clustered distantly from the CG area.

Only the t_1 sample obtained from horse 89 showed some signs of movement towards the CG area (**Figure 3B**). At t_2, data points representing the samples of horses 33, 87 and 89 clustered within the CG area, indicating the onset of microbiome recovery that was accompanied by a notable SDI increase (5.76, 6.02, 5.76).

SSG: The initial data point representing the t_0 sample of horse 45 (SDI 6.66) clustered near the area covered by the CG. While the corresponding data point at t_1 indicated an increase of gut microbiome disturbance accompanied by an SDI decrease to 5.97, the t_2 sample (SDI 6.15) clustered within the area framed by data points of the CG samples, once again indicating the onset of microbiome recovery.

However, some equine patients deviated from the aforementioned common temporal trajectory: The data points of horse 41 (SSG), for instance, indicated a considerable microbiome disturbance at t_0 (SDI 5.05) followed by a brief relative recovery at t_1 (SDI 5.99). Then, the data point of horse 41 shifted towards the opposite direction at t_2 (**Figure 3A**), a reversal that is accompanied by an SDI decrease to 5.59.

Taken together, the control samples seemed to represent an overall beneficial structure and composition of equine gut microbiota, since all patients' samples associated with an SDI > 5.8 clustered near or within the area covered by these samples, while samples associated with lower SDIs clustered elsewhere (**Figure 3** and **Supplemental Table 1**). Moreover, although a common temporal trajectory from hospital admission towards discharge was notable for the gut microbiota of most participants in both study groups, the temporal patterns of some horses deviated from these, emphasizing the individual nature of the GIT microbiome recovery process.

ii) Evaluation of gut microbiota alterations among horses receiving different PAP regimens after colic surgery

To gain insights into the putative effect of the different PAP regimens on the equine gut microbiota, log 2-fold changes (log2FC) were calculated based on OTU count changes on bacterial family level between sampling timepoints. Overall, the most

prominent log2FC between t_0 and t_1 in the SSG were noticed for *Bacteroidaceae* (+5.16; p<0.05), *Enterobacteriaceae* (+3.99; p<0.05) and *Pseudomonadaceae* (+4.41; p<0.05). Among the most prominent log2FC between t_0 and t_1 in samples of the 5DG were *Bacteroidaceae* (+2.96; p<0.05) and *Pseudomonadaceae* (+3.33; p<0.05). In order to further investigate the relevance of the aforementioned observations and to better assess the influence of the colic syndrome complex as well as antibiotic treatment on the abundances of specific bacterial families in the GIT of horses throughout this study, further log2FC values were determined based on the abundances calculated for the CG.

Divergence among microbiota composition on family level considering a baseline defined by the CG samples

Since the PCA confirmed the eligibility of the CG samples with respect to an overall favourable equine gut microbiota structure and composition, we determined the median relative abundance for bacteria (family level) among the CG samples. To enhance identification of relevant changes associated with microbiome disturbance among samples belonging to both study groups, the median for each bacterial family was calculated using the t_0 CG samples as a baseline. Then, log2FC for each sample/timepoint/study group/ were determined to pin-point variation that might have been overlooked by simply investigating mean abundances. In order to compensate for individual differences between the equine patients (i.e. age, diet, medical history, severity and duration of the acute colic episode, housing and social contacts), an interval comprising at least 85% of the SSG and 5DG samples was defined (**Figure 4**; log2FC +/- 2.5).

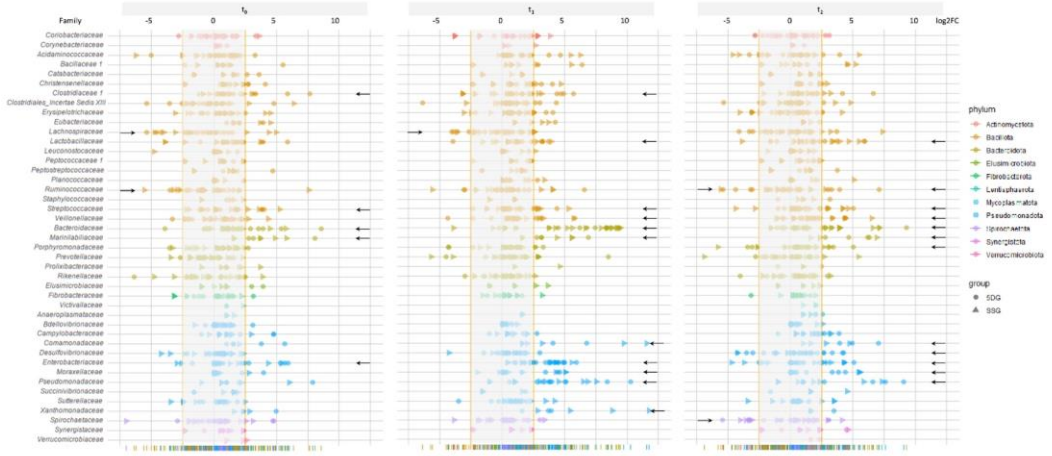

Figure 4| Changes of the fecal microbiome structure and composition during the study period

Illustration of log2FC abundance changes for bacterial families in each sample of both study groups with respect to a baseline calculated from the control group samples at t_0. To acknowledge individual variation and to enhance the chances of overall trend detection, an interval comprising the majority of samples (85% at t_0; log2FC of 2.5) was calculated, illustrated as the blurred area per timepoint. Individual values that were zero (i.e. no abundance difference compared to the control) were not shown for better clarification. Data points belonging to samples from the SSG are marked by a triangle and those from the 5DG by a dot, while deviation from the baseline within at least 5 samples are indicated by a black arrow.

To identify the most deviations with high potential relevancy, further in-depth explorative analysis was restricted to bacterial families associated with at least five samples (SSG & 5DG) that clustered beyond the 85% interval (**Figure 4**, black arrows indicate bacterial families fulfilling the restriction criteria).

Directly at hospital admission and before any antibiotics were administered (t_0), six samples showed log2FC between 2.7 and 5.2 for *Streptococcaceae*, seven samples for *Bacteroidaceae* (log2FC between 2.9 and 8.6), seven samples for *Marinilabiliaceae* (log2FC between +2.8 and +8.1) and six samples for *Enterobacteriaceae* (log2FC between +2.8 and +5.9). Overall, lower values were noted for *Lachnospiraceae* (six

samples, log2FC between -3.1 and -5.4) and *Ruminicoccaceae* (five samples, log2FC between -2.7 and -3.5) (**Figure 4**, t_0; indicated by black arrows).

At t_1, increasing log2FC fulfilling the above mentioned criteria were noticed for *Clostridiaceae*, *Lactobacillaceae*, *Streptococcaceae*, *Veillonellaceae*, *Marinilabiliaceae* and *Xanthomonadaceae*, but most prominently for *Bacteriodaceae* (22 samples, log2FC between +3.7 and +9.5), *Comamonadaceae* (five samples, log2FC between +3.2 and +11.6), *Enterobacteriaceae* (17 samples, log2FC between +2.7 and +5.9), *Moraxellaceae* (6 samples, log2FC between +3.5 and +5.1) and *Pseudomonadaceae* (15 samples, log2FC between +2.8 and +10.3) (**Figure 4**). *Lachnospiraceae*, on the other hand, yielded log2FC between -2.7 and -4.0 in five samples.

At t_2, deviation from the baseline interval (log2FC ≥+/- 2.5) defined for bacterial families were recognized for *Bacteriodaceae* (eleven samples, log2FC between +2.8 and +9.3), *Enterobacteriaceae* (eight samples, log2FC between +2.6 and +5.1) and *Lactobacillaceae* (nine samples, log2FC between +2.8 and +5.9) followed by *Streptococcaceae*, *Veillonellaceae*, *Marinilabiliaceae*, *Comamonadaceae*, *Moraxellaceae* and *Pseudomonadaceae* (**Figure 4**). Overall, a shift back to the baseline was directly recognizable for most of the displayed bacterial families.

Taken together, OTU abundances of many bacterial families deviated considerably from those associated with samples of the CG (t_0). In addition, overall deviation increased at t_1 and decreased at t_2, a result that is clearly in congruence with the SDI values and the trajectory pattern displayed by PCA (**Figure 3A-D**). Of note, clear differences between the study groups were not noticed in **Figure 4**. These results confirmed that although a common temporal trajectory pattern was recognizable, deviation of individuals contributed largely to the overall variances.

iii) Hospitalization, surgery and administration of PAP is accompanied by a predominant converged trajectory pattern of *Escherichia* and *Bacteroides*

At t_1, deviations of *Bacteroidaceae* and *Enterobacteriaceae* stood out considering the baseline defined by the CG samples (**Figure 4**). Although relative abundances of OTUs within an individual sample depended on each other, we noticed stunning trajectories of OTUs assigned to *Bacteroides* and *Escherichia*, the predominating genera of the above-mentioned families within our sample set **(Figure 5**).

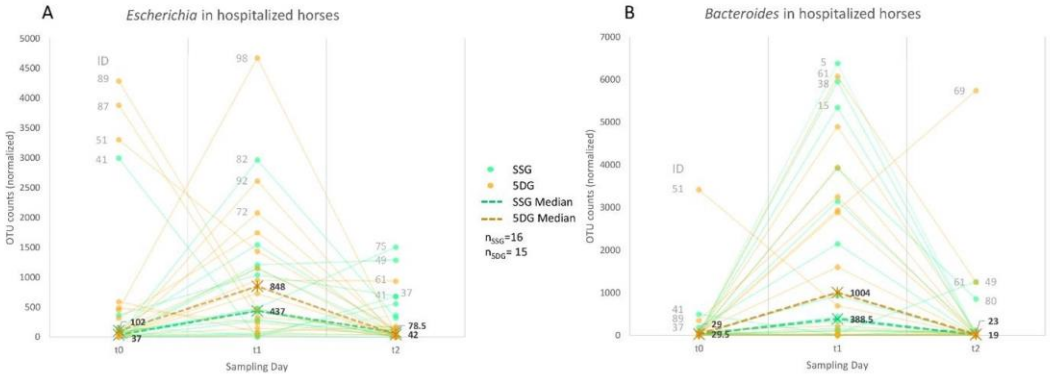

Figure 5| Abundance trajectories of OTUs assigned to the genera Escherichia and Bacteroides

Charts showing a trend line based on normalized OTU counts per sample/study group and timepoint. A dashed line indicates the median per group and sampling day.

Apart from four horses (89, 87, 51 and 41), most study participants showed limited OTU counts ($median_{SSG}$= 37; $median_{5DG}$= 102) classified as *Escherichia* (genus level) at t_0, while the overall counts increased at t_1 (**Figure 5A**), with a median OTU counts of 437 (SSG) and 848 (5DG) in samples of both groups, respectively (SSG vs. 5DG, t_0: p=0.14, t_1: p=0.086, t_2: p=0.95). This difference was significant between the SSG and 5DG from t_1 to t_2 (differences in counts, SSG vs. 5DG, t_0 to t_1: p=1, t_1 to t_2: p=0.0019). Of note, both study groups showed a decline to almost similar counts assigned to *Escherichia* at t_2 ($median_{SSG}$= 79 OTU counts; $median_{5DG}$= 42 OTU counts).

Interestingly, the hospitalized horses demonstrated a highly similar trajectory pattern for OTU counts assigned to the genus *Bacteroides*. Besides a single exception (horse 51, 5DG), both study groups had comparable counts for *Bacteroides* ($median_{SSG}$ =

29.5 OTU counts, $median_{5DG}$ = 29 OTU counts) at t_0 (SSG vs. 5DG, t0: p=0.67, t1: p=0.45, t2: p=0.69). Changes in differences were not significant across time points (differences in counts, SSG vs. 5DG, t_0 to t_1: p=0.89, t_1 to t_2: p=0.92).

At t_1, the OTU counts for *Bacteroides* doubled in abundance within the 5DG compared to the SSG ($median_{SSG}$ = 389, $median_{5DG}$ = 1004 OTU, p = 0.45). However, the *Bacteroides* count level decreased once more at the last day of sampling (t_2) in both groups ($median_{SSG}$= 19; $median_{5DG}$= 23, p=0.69).

In summary, the abundance counts of both *Escherichia* and *Bacteroides* seemed to be associated with a more pronounced increase among samples representing the 5DG, i.e. horses that received the long-term PAP, compared to horses belonging to the SSG at t_1. Correlation analysis revealed no obvious relationship between the two genera in the sample set investigated.

4 Discussion

In the present study, we explored temporal alterations of the gut microbiota composition and the extent of its perturbation among hospitalized horses subjected to colic surgery that received either a short-term (SSG) or a 5-day course (5DG) of P/G PAP. Of note, comparison of fecal microbiota profiles of horses belonging to different cohorts or even between distinct individuals requires immense caution, since the intestinal microbiome is easily affected by external factors, including - but not limited to - exercise (Górniak et al., 2021), transport, fasting (Schoster et al., 2016) and diet (Al Jassim, 2006;Mshelia et al., 2018). In addition, Antwis and colleagues recently examined how spatial and social interactions affected the gut microbiome composition of semi-wild Welsh Ponies, revealing that up to 52.6% of the observed variation is attributable to individual variation (Antwis et al., 2018).

At first, we determined the predominating microbial phyla in the sample set, revealing *Bacteroidota* (38%), *Bacillota* (33%) and *Verrucomicrobiota* (11%) as the top three main phyla at hospital admission (t_0) and in the CG (**Figure 2**). This result is in line with previous reports defining the *Bacteroidota* and *Bacillota* as the major phyla of the core bacterial community in equines (Morrison et al., 2018;Edwards et al., 2020).

Gut microbiota diversity of hospitalized horses receiving colic surgery and P/G PAP elicits a predominant trajectory pattern from hospital admission to discharge

As summarized previously, most of the current clinical studies regarding the effects of antibiotics on the gut microbiome have been cross-sectional, while interventional or longitudinal approaches and comparisons to treatment-naive but diseased control groups are often missing (Zimmermann et al., 2021). As a result, it is difficult to differentiate between disease-mediated, drug-related hospitalization effects (Zimmermann et al., 2021), an aspect that clearly is a limitation with respect to the discussion of putative drug-related effects in the current study. Although the isolated

and exact impact of the P/G regimen on the gut microbiota of horses participating in this study remains unknown due to the fact that our study was not an animal trial but a real-world scenario, recent research on the effect of parental administration of P/G on the developing infant gut microbiome clearly revealed an impact on Shannon diversity and overall gut microbiota composition (Reyman et al., 2022). However, at hospital admission and before antibiotic treatment and hospitalization (t_0), the mean alpha-diversity measured by SDIs revealed strong signals of an already compromised bacterial biodiversity in samples representing the SSG and the 5DG compared with those of the CG (**Figure 3D**), although only the latter difference was found to be significant. A loss of species diversity seems to be among the prominent characteristic of a disturbed gut microbiota (Ramirez et al., 2020). At hospital admission, a decrease in bacterial richness and diversity accompanied with a greater inter-individual variability was reported for horses admitted due to colic compared with horses presented for elective surgical procedures or even healthy horses (Stewart et al., 2018;Park et al., 2021). A similar trend was detected in the current study, demonstrating a considerable range of variation between microbiota profiles and microbiome perturbations among horses suffering from colic compared to horses free of abdominal pain or otherenteral disorders.

A previous study by Costa et al. examined the effects of intramuscular administration of procaine penicillin and ceftiofur sodium, and oral trimethoprim sulfadiazine on the fecal microbiome of healthy horses (Costa et al., 2015). Although the administration route and duration, the cohort under investigation and the combination of antibiotics (P/G) differed in the present study, general similarities should be mentioned. The strongest perturbation of the microbiome was recognized directly after the final course of antibiotics, including a strong effect on the microbial community membership (Costa et al., 2015).

The most prominent peak of microbiome disturbance is characterized by significant SDI decreases at t_1 (i.e. three days after surgery/hospitalization) (**Figure 1**). The

considerable inter-individual distances between the microbiota profiles of 5DG horses at t_0 have diminished immensely at t_1, indicating a similar and consistent effect of the long-term PAP regimen on the gut microbiota composition that is specific for the long-term P/G PAP.

This observation is in line with the results of a recent study investigating the effects of commonly used antibiotics on the gut microbiome of healthy human volunteers before and after treatment, where the authors revealed drug-specific trajectories through the PCA space over time (Anthony et al., 2022). In strict contrast to the PCA of the 5DG in the present study, data points of the SSG showed increased inter-individual differences through the PCA space at t_1, indicating the lack of a common selective factor. Therefore, other more distinguishing factors associated with the distinct individual such as appetite, stress and/or pain, environmental bacteria or even the onset of GIT function and/or microbiome recovery (reviewed in (Kauter et al., 2019)) might be mirrored here.

At t_2, both study groups showed clear signs pointing towards the onset of microbiome recovery with respect to a rise of both SDI_{mean}, with most participants seeming to have already regained the baseline condition, since the difference between t_0 and t_2 lacked statistical differences (**Figure 3**). Interestingly, in a study of germ-free mice, the recovery of the gut microbiome after antibiotic treatment strongly depended on diet, community context and environmental reservoirs (Ng et al., 2019). The authors demonstrated that a reduction of environmental reservoirs impaired the process of microbiota recovery (Ng et al., 2019). This fact not only emphasizes the overall importance of the actual environmental bacteria in the immediate vicinity of hospitalized horses during microbiome recovery, but it also highlights the susceptibility of the equine gut microbiota to spatio-temporal local spreads of hospital-associated pathogens, explicitly including ESBL-EC, leading to worrisome carriage rates, as previously reported (Kauter et al., 2019).

Since studies on equine gut microbiomes are currently limited (Ang et al., 2022), it seems reasonable to assume that some general effects occur across different mammalian species: Exposure of gut microbiota to antibiotics or their still active metabolites reduces its diversity, while the absence of antibiotics leads to an altered state that is either transient or permanent (Costa et al., 2015;Ramirez et al., 2020;Arnold et al., 2021). Moreover, the effect might not be limited to a reduced microbial diversity, since a recent study on healthy human volunteers showed a worrisome increase of virulence- and resistance associated-factors immediately after antibiotic treatment (Palleja et al., 2018). Further studies on the equine gut metagenome (Ang et al., 2022) after exposure to different antibiotics are required to gain more insights with respect to spatial trends of virulence- and resistance gene occurrences and abundances.

Alterations of the equine gut microbiota in the SSG and 5DG

At first, we analyzed the significant log2FC of the SSG and the 5DG. Samples of horses belonging to both study groups were associated with a significant abundance increase for *Bacteroidaceae*, *Enterobacteriaceae* and *Pseudomonadaceae* from t_0 to t_1. A previous study that examined changes in the equine fecal microbiome during hospitalization because of colic reported a similar increase for *Bacteroides* (Stewart et al., 2021). The increased abundances noticed for *Enterobacteriaceae* at t_1 in both groups might enhance the risk of developing surgical site infections (SSI) for the equine patients, especially since multidrug-resistant Enterobacterales were often reported within horse clinics (Apostolakos et al., 2017;Walther et al., 2018;Shnaiderman-Torban et al., 2020), respectively in cases of SSI of horses subjected to laparotomy (Isgren et al., 2017;Dziubinski et al., 2020). Furthermore, significant changes were recognized regarding *Pseudomonadaceae*, a bacterial family that has been described as indicator of intestinal microbiome alterations in the human gut since an abundance increase seems to be associated with various gastrointestinal diseases (Alam et al., 2020;Chamorro et al., 2021).

Secondly, we explored the probable influence of the acute disease and other factors on the fecal microbiota. For this, we evaluated the abundance of different bacteria in fecal samples of both study groups compared to the control group at t_0, revealing considerable deviation of OTUs belonging to 15 different bacterial families (**Figure 4**). Overall, a common temporal trajectory pattern was recognizable regarding *Bacteroidaceae*, *Enterobacteriaceae* and *Pseudomonadota*, with a strikingly increased proportion in almost all t_1 samples and decreasing, but still above-baseline level, OTU counts at t_2. In addition, we observed a reduced frequency of *Lachnospiraceae* for the horses diagnosed with colic, compared to the horses belonging to the CG, which is in line with recent findings (Stewart et al., 2019). Moreover, a reduction of *Lachnospiraceae* was previously described for horses suffering from enteral disorders (Costa et al., 2012;Weese et al., 2015).

Although differences between the study groups were limited, we observed interesting differences with respect to the effect of the distinct P/G PAPs on the microbiota. Horses that received the 5-day course of PAP showed the highest loss of inter-individual diversity (**Figure 3B**, t_1) which is clearly accompanied by the most prominent expansion of *Escherichia* (**Figure 5**). Further studies including metagenomic data are needed to reveal the particular dependences here.

Gut microbiome perturbances are associated with increased *Escherichia* and *Bacteroides* abundances

Since penicillin and gentamicin were administered parenterally (Stöckle et al., 2021), the effect on the gut microbiome in general was expected to be lower than in cases when oral antibiotics where administered (Zhang et al., 2013). In the present study, however, the combined influences of (intestinal) illness, hospitalization, surgery and administration of P/G PAP showed a converged relative abundance trajectory of the genera *Escherichia* and *Bacteroides* over time, as demonstrated in the results (**Figure 5**). A recent review on the effects of distinct antibiotic classes on the GIT microbiome in humans highlighted an increase of *Enterobacteriaceae* and *Bacteroidaceae* after

treatment with β-lactams in general (Patangia et al., 2022), which seems strikingly in line with the results presented here.

The obligatory anaerobic genus *Bacteroides* exclusively growing in the GIT of mammals is a major research focus of gut microbiology (reviewed (Wexler and Goodman, 2017)). Since intrinsic resistance is reported for *Bacteroides* spp. (Pumbwe et al., 2006;CLSI, 2020) at least an impact of a treatment-associated selective advantage can be assumed when considering the relative abundance increase among most of the fecal samples of both study group participants at t_1 (**Figure 5**), in particular for the 5DG.

Although many *Bacteroides* species play a crucial role in degrading polysaccharides of a plant-based diet (Pereira et al., 2021;Cheng et al., 2022), the specific importance and role of distinct *Bacteroides* species in hindgut fermentation has not been investigated yet. More research on the subject including metabolomic profiles gained from metagenome sequencing projects is clearly required to shed more light on this subject.

Apart from outliers (four horses, **Figure 5**), most of the horses participating in the present study revealed a low relative abundance of *Escherichia* at hospital admission. This observation is in line with the previously reported increase of ESBL-EC carriage rates among patients of both study groups over time, i.e. from hospital admission to discharge (**Supplementary Table 1**), that indicated local spread of, for instance, distinct ESBL-EC clonal lineages, as reported previously (Kauter et al., 2021). We further speculate that during P/G PAP, ESBL-EC had a selective advantage in the GIT of horses and therefore proliferate, resulting not only in increased carriage rates (Kauter et al., 2021), but also in unavoidable environmental contamination via feces-contaminated litter. Since the immediate environment is among the main sources of GIT-associated bacteria during microbiome recovery (Ng et al., 2019), environmental sources seem to play an overwhelming role with respect to ESBL-EC carriage rates. Since the specific needs and surroundings of hospitalized horses (bedding, boxes,

floor surfaces, reviewed in (Gehlen et al., 2020) differ immensely from those of small animal or even human patients, the environment of equine clinics seems to play an important role in ESBL-EC accumulation and spread, including strains known for their pathogenic potential in humans and animals, i.e. isolates belonging to sequence type (ST)10 and ST410 (Kauter et al., 2021).

Taken together, the spread of *E. coli*, especially ESBL-EC in horse clinics, seems to be promoted by i) the selective advantage of these bacteria towards β-lactam antibiotics and ii) the fact that the fecal microbiota structure is re-modelled by other factors occurring during the course of hospitalization, such as ingestion by food, contact to environmental sources or transmission via healthcare workers.

Conclusion

In the present study, we investigated the influence of two different PAP regimens (SSG vs. 5DG) in horses diagnosed with colic syndrome that were subjected to surgery with regard to changes of their gut microbiota composition. Colic surgery and PAP drive the equine gut microbiome towards dysbiosis and reduced biodiversity that is accompanied by a 10-fold increase of samples positive for ESBL-producing Enterobacterales (Kauter et al., 2021) and an abundance increase for Enterobacteriaceae. Further studies are needed to reveal the most important local sources of the resistant bacteria (i.e. environment, food, contacts) and factors promoting the inclusion of ESBL-producing Enterobacterales in the equine gut microbiota.

Availability of data and materials

The established workflow was implemented in Python and is freely available under GPLv3 license (https://github.com/SiWolf/Meta16s/). The repository includes further downstream analysis and visualization scripts in R, as well as an associated Conda environment for reproducibility. Raw 16S rRNA gene sequences were submitted to NCBI and are stored within BioProject PRJNA906950.

Authors' contributions

BW, AL-B, LHW and HG designed the project. AK, SDS, HG, and BW conceived and designed the experiments. AK, DK, SDS, JB and AL-B performed laboratory analysis. CB and SF sequenced the samples. AK, SAW, AL-B, BW and TS analyzed the data. AK, SAW, and BW wrote the first draft. AM, NE, RK and SG helped to draft the manuscript and contributed to the discussion. All authors have read and approved the final draft of the manuscript.

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Ethics

According to the German regulation authorities for research with animal subjects, the comparison of two PAP regimens does not require explicit approval (Landesamt für Gesundheit und Soziales, Berlin, 18.04.2017). Written owner's consent regarding the involvement of their horses in the study was obtained directly during the hospital admission process (Stöckle et al., 2021).

Conflict of Interest

The authors declare that they have no competing interests.

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Abbreviations

5DG : 5-day course of peri-operative antibiotic prophylaxis group

Bp: base pair

CG: control group

EC: *Escherichia coli*

ESBL: extended-spectrum β-Lactamase

G: gentamicin

GIT: gastrointestinal tract

i.e.: id est

Log2FC: log_2 fold change

MDR: multi-drug resistance

OTU: Operational Taxonomic Units

P: penicillin

PAP: perioperative antibiotic prophylaxis

PCA: principal component analysis

SDI: Shannon diversity index

SSG : single-shot of peri-operative antibiotic prophylaxis group

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Abbildungsverzeichnis

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Eigenständigkeitserklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbstständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

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Anne Kauter

Verzeichnis aller Veröffentlichungen

Wissenschaftliche Artikel

Anne Kauter, Silver A. Wolf, Julian Brombach, Antina Lübke-Becker, Dania Kannapin, Corinna Bang, Sören Franzenburg, Sabita D. Stoeckle, Sebastian Günther, Lothar H. Wieler, Heidrun Gehlen, Torsten Semmler, Birgit Walther: Antibiotic prophylaxis and hospitalization of horses subjected to median laparotomy: gut microbiota trajectories and expansion of the genera Escherichia. Submitted to *Front. Microbiol.* 5 (2023) [Preprint] doi: 10.1101/2023.05.24.542119

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