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DISSERTATION

Untersuchungen zur antimikrobiellen und immunmodulierenden Wirksamkeit definierter Moleküle in *Campylobacter jejuni*-infizierten Mäusen

Investigations on the antimicrobial and immune-modulating properties of defined molecules in *Campylobacter jejuni* infected mice

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Eine detaillierte Beschreibung des Beitrags an den erfolgten Publikationen ist der Anteilserklärung zu entnehmen (siehe S. XXXII-XXXIII).

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Abkürzungsverzeichnis

CBA	engl. Cytometric Bead Array
CFU	engl. colony forming units
GBS	Guillain-Barré-Syndrom
HPF	engl. high power fields
IBD	engl. inflammatory bowel diseases
IFN	Interferon
IL	Interleukin
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MCP	engl. monocyte chemoattractant protein
MFS	Miller-Fisher-Syndrom
MLN	engl. mesenteric lymph nodes
NO	Stickstoffmonoxid
PBS	engl. phosphate-buffered saline
PLC	Placebo
RA	reaktive Arthritis
SPF	spezifisch pathogenfrei
TBST	engl. Tris-buffered saline with Tween20
Th	T-Helfer
TLR	Toll-Like Rezeptor
TNF	Tumornekrosefaktor

Abstrakt (Deutsch)

Die Campylobacteriose ist aktuell die häufigste meldepflichtige bakterielle Durchfallerkrankung in der Europäischen Union und in den Industrienationen weltweit. Durch den Konsum kontaminiertem Fleisch wird die Erkrankung aus der Nutztierhaltung über die Fleischproduktion auf den Menschen übertragen. Zusätzlich entwickelt *Campylobacter* aktuell gegen klinisch wichtige Antibiotika zunehmende Resistenzen. Da keine Daten zur Wirksamkeit alternativer Wirkstoffe gegen *Campylobacter* vorliegt, ist die Entwicklung neuer Strategien für die Prophylaxe und Therapie der Campylobacteriose ein grundlegendes Anliegen der medizinischen Forschung weltweit. In unseren präklinischen Interventionsstudien haben wir die antimikrobiellen und immunmodulatorischen Eigenschaften von Carvacrol, Ascorbinsäure (Vitamin C) und 25-OH-Cholecalciferol (Vitamin D) gegen die akute Campylobacteriose im murinen Infektionsmodell untersucht.

Sekundär abiotische Interleukin-10-defiziente Mäuse, die mit Carvacrol und Vitamin C vorbehandelt worden waren, wiesen eine geringere Belastung mit *C. jejuni*-Bakterien im Colon auf und zeigten im Vergleich zu den Placebo-Kontrolltieren eine signifikant verminderte Symptomatik der Campylobacteriose. Diese Besserung war auf eine Reduktion der Apoptose und der pro-inflammatorischen Immunreaktionen im Darm zurückgeführt werden.

Auch die perorale prophylaktische Behandlung mit Vitamin D bewirkte eine signifikante Besserung der Campylobacteriose, die ebenfalls auf immunmodulatorische Wirkungen auf die durch *C. jejuni* induzierte Enterokolitis zurückgeführt werden konnten. Die in unserem standardisierten präklinischen Mausmodell für die akute humane Campylobacteriose beobachteten prophylaktischen und therapeutischen Wirkungen der Moleküle gegen die schwere, durch *C. jejuni*-induzierte Infektionskrankheit zeigt erstmals, dass die Anwendung definierter Vitamine und pflanzlicher Verbindungen mit antimikrobiellen und/oder entzündungshemmenden Eigenschaften eine vielversprechende neue Option für die Behandlung der Campylobacteriose darstellen. Die antimikrobiellen Wirkungen weisen darauf hin, dass die Substanzen auch für eine Verringerung der Kolonisierung von *C. jejuni* im Darm von Nutztieren geeignet sein könnten.

Abstract (English)

Campylobacteriosis is currently the most common bacterial gastrointestinal infection worldwide. By consuming contaminated meat, the bacteria can transmit from livestock animals to humans. In addition, *Campylobacter* develop currently increasing resistance to clinically important antibiotics. Since no data are available on the effectiveness of alternatively active substances against *Campylobacter*, the development of new strategies for the prophylaxis and therapy of campylobacteriosis is a fundamental concern of medical research worldwide. In our preclinical intervention studies, we examined the antimicrobial and immunomodulatory properties of carvacrol, ascorbic acid (vitamin C) and 25-OH-cholecalciferol (vitamin D) against acute campylobacteriosis in a murine infection and inflammation model.

Secondary abiotic interleukin-10 deficient mice that had been pretreated with carvacrol and vitamin C experienced less exposure to *C. jejuni* bacteria in the colon and showed significantly reduced symptoms of campylobacteriosis compared to the placebo control animals. This clinical improvement was due to a reduction in apoptosis and pro-inflammatory immune responses in the intestine.

Peroral prophylactic treatment with vitamin D also brought about a significant improvement in campylobacteriosis, which could also be attributed to immunomodulatory effects on enterocolitis induced by *C. jejuni*. The prophylactic and therapeutic effects of the molecules against the severe infectious disease induced by *C. jejuni*, observed in our standardized preclinical mouse model for acute human campylobacteriosis, showed for the first time that the use of defined vitamins and plant compounds with antimicrobial and/or anti-inflammatory properties is a very promising new option for the treatment of campylobacteriosis. The antimicrobial effects indicate that the substances could also be suitable for reducing the colonization of *C. jejuni* in the intestines of farm animals.

1. Einführung

1.1 Die *Campylobacter*-Infektion

Die Campylobacteriose ist aktuell die häufigste meldepflichtige bakterielle Durchfallerkrankung in der Europäischen Union und in den Industrienationen weltweit. Die kontinuierlich steigenden Fallzahlen der Campylobacteriose, die die Meldungen der Salmonellosen übertreffen, stellen ein aktuelles globales Problem dar [1]. Unter mehr als 30 *Campylobacter*-Arten und -Subspezies sind *C. jejuni* und *C. coli* die häufigsten Subpopulationen, die Infektionen beim Menschen hervorrufen, wobei die meisten gemeldeten Infektionen durch *C. jejuni* verursacht werden [2].

Die *C. jejuni*-Bakterien sind Gram-negative, spiralförmig gekrümmte Stäbchen, die durch ihre Flagellen sehr beweglich sind [3]. Sie wachsen in einem Temperaturbereich zwischen 37 und 42°C unter mikroaerophilen Bedingungen. *C. jejuni* kommt in Oberflächengewässern vor und ist Teil der natürlichen Darmmikrobiota einer Vielzahl von Wildtieren und Nutztieren. Die Infektion beim Menschen wird in den meisten Fällen durch unzureichend gegartes Geflügelfleisch verursacht [4-6]. Die Resultate einer neuen Studie belegen die Übertragung von *C. jejuni* von Hühnern auf Menschen und unterstreicht die Tatsache, dass der Konsum von Geflügel als Hauptquelle menschlicher *C. jejuni*-Infektionen gilt [7]. In zahlreichen Studien wurden *C. jejuni* und *C. coli* aber auch in Erbsen [8], Spinat und Radieschen [9] nachgewiesen. Die beobachtete Kontamination von Gemüse kann durch deren Bewässerung mit kontaminiertem Wasser und/oder die Verwendung natürlicher Düngemittel erfolgen [10]. Tatsächlich infizieren sich Menschen in den meisten Fällen durch den Verzehr von nicht gekochtem Fleisch kontaminierter Nutztiere oder durch die Aufnahme von Rohmilch und Oberflächenwasser, die *C. jejuni* enthalten [3,5].

Je nach *Campylobacter*-Stamm und dem Immunstatus der infizierten Menschen kann das Krankheitsbild variieren. Die Patienten können einen hochakuten und schweren Symptomkomplex entwickeln, der von wässrigem Durchfall und/oder Bauchkrämpfen bis zu schwerer Campylobacteriose reicht, gekennzeichnet durch blutigen entzündlichen Durchfall und systemische Entzündungsreaktionen, einschließlich Fieber [4]. Normalerweise ist die Infektion selbstlimitierend und dauert mehrere Tage bis maximal zwei Wochen [5]. Gemäß den Empfehlungen der S2k-Leitlinie "Akute infektiöse Gastroenteritis“ sollte bei einer *C. jejuni*-Infektion keine antibiotische Behandlung durchgeführt werden, wenn eine Besserung der klinischen Symptome festgestellt wurde [11]. Nach einer Infektion mit *C. jejuni* können sich postinfektiöse Komplikationen wie das Guillain-Barré-Syndrom (GBS), das Miller-Fisher-Syndrom (MFS), die reaktive Arthritis (RA) und chronisch entzündliche Darmerkrankungen (engl. inflammatory bowel diseases, IBD) entwickeln [5]. Bei schwerem Krankheitsbild, fehlender klinischer Besserung oder Immunsuppression sollte unter Beachtung der

Resistenztestung eine antibiotische Behandlung mit Azithromycin (bevorzugt) oder Ciprofloxacin durchgeführt werden [11].

Klinische Studien ergaben, dass Strukturen an der Zelloberfläche von Gram-negativen Bakterien (zum Beispiel Lipopolysaccharid-Struktur; LPS) das Krankheitsbild signifikant beeinflussen können [12]. Die Lipooligosaccharid (LOS) -Strukturen in der äußeren Zellmembran von *C. jejuni*, vor allem in sialylierter Form, spielen sowohl bei der Schwere der Campylobacteriose durch die Hyperaktivierung des angeborenen Immunsystems als auch bei der Entwicklung postinfektiöser Komplikationen eine wichtige Rolle [13].

Das LOS der *C. jejuni*-Bakterien besteht aus der hydrophoben Lipid-A-Einheit und einem Oligosaccharid mit einem konservierten inneren und einem variablen äußeren Kern [14]. Trotz der Abwesenheit des für LPS charakteristischen O-Antigens ist die Struktur von *C. jejuni*-LOS variabel [5,14]. Verantwortlich dafür sind die kodierenden Gene für LOS, die an einem hypervariablen Ort mit circa 20 identifizierten Klassen lokalisiert sind [15]. Molekulare Analysen zeigten, dass die Unterschiede in der LOS-Struktur auch durch horizontalen Gentransfer zwischen verschiedenen *C. jejuni*-Stämmen übertragbar sind [16]. Die Mehrheit der *C. jejuni*-Stämme produzieren sialyliertes LOS, dessen Struktur den neuronalen Gangliosiden des Menschen sehr ähnlich sind [17]. In klinischen Studien wurde gezeigt, dass die Infektion mit *C. jejuni*-Stämmen, die die sialylierten LOS-Typen A, B und C produzieren, signifikant mit einem schweren Verlauf der Enteritis assoziiert sind, der wiederum das Risiko für die Entwicklung der oben genannten Autoimmunerkrankungen erhöht. Für die Immunpathogenese des GBS wird daher zurecht vermutet, dass gegen *C. jejuni*-LOS gerichtete Antikörper mit peripheren Nerven reagieren und die Krankheit so auslösen [18].

Die spezifische Bindung der Lipid-A-Einheit des *C. jejuni*-LOS sowie des LPS durch den Toll-Like-Rezeptor (TLR) -4 des angeborenen Immunsystems löst eine Signalkaskade aus, die beim Menschen besonders stark ausgeprägt ist. So sind Mäuse und Hühner 10.000- bzw. 100.000-fach resistenter gegen LOS und LPS als der Mensch [19].

Beim Menschen führt die Bindung von *C. jejuni*-LOS an TLR4 über die Produktion von inflammatorischen Zytokinen zu einer Hyperaktivierung des angeborenen Immunsystems, die über die Akkumulation von Granulozyten im Darmgewebe die Symptomatik der schweren Enteritis bewirkt. Die Entwicklung einer menschlichen *C. jejuni*-induzierten Enterokolitis tritt in verschiedenen Stadien auf, die durch unterschiedliche Immunantworten gekennzeichnet sind [5,20]. Im Frühstadium (in der ersten Woche während einer akuten Enterokolitis) werden pathologische epitheliale Veränderungen vorwiegend in distalen Teilen des Dickdarms durch eine massive Anreicherung von Makrophagen und neutrophilen Granulozyten in Krypten und in der Lamina propria verursacht. Im späteren Stadium (in der zweiten Woche) sind die Entzündungssymptome weniger ausgeprägt, und die Regeneration der betroffenen Epithelien beginnt. Nach zwei Wochen sind die entzündlichen Veränderungen dann fast vollständig

abgeklungen. Interessanterweise wurden *C. jejuni*-Bakterien unabhängig vom Infektionsstadium immunhistochemisch in Kolonbiopsien nachgewiesen, die von Patienten mit *C. jejuni*-positiven Stuhlkulturen stammen [20]. Diese Befunde sind für das Verständnis der molekulare Immunpathologie der Campylobacteriose und damit für die Behandlung von grundlegender Bedeutung.

1.2 Sekundär abiotische IL-10$^{-/-}$ Mäuse als *C. jejuni* Infektionsmodel

Seit der Entdeckung von *Campylobacter*-Arten als Darmpathogene (wahrscheinlich durch Theodor Escherich Ende des 19. Jahrhunderts [21]) wurde die Erforschung der Immunpathogenese der Campylobacteriose durch das Fehlen zuverlässiger experimenteller *in vivo*-Modelle erschwert [22]. Diverse Wirbeltierarten, wie Ferkel und Frettchen, die ähnlich LPS/LOS-empfindlich wie der Mensch sind, wurden erfolgreich für die Induktion der Darmentzündung durch *C. jejuni* verwendet; aber die begrenzte Verfügbarkeit genetisch standardisierter Tiere schränkte die breite Verwendung dieser Modellorganismen für die Campylobacteriose-Forschung ein [5,19,22].

Die physiologischen und genetischen Ähnlichkeiten zwischen Mäusen und Menschen haben Mäuse als Modellorganismus für das Studium der Humanbiologie ins Rampenlicht gerückt. Jedoch sind die Mäuse gegen LPS/LOS 10.000-fach resistenter als Menschen und reagieren deshalb auf die Kolonisierung des Darms mit *C. jejuni* nicht mit Entzündungsreaktionen, die denen des Menschen ähnlich sind [5,19].

Um ein klinisches Mausmodell für die schwere Campylobacteriose des Menschen zu etablieren, nutzten wir die bereits von Mansfield et al. [23] und Lippert et al. [24] beschriebenen Interleukin 10-defizienten (IL-10$^{-/-}$) Mäuse, die wegen des Fehlens von IL-10 gegen LOS/LPS sensibel sind [25]. Die IL-10$^{-/-}$ Mäuse entwickeln nach Infektion mit *C. jejuni* eine schwere Enteritis, die klinische und histopathologische Schlüsselmerkmale der menschlichen Campylobacteriose widerspiegelt [26]. Der Mangel an dem entzündungshemmenden Zytokin IL-10 verstärkt die TLR4-abhängigen angeborenen Immunantworten. Dies führt zur Aufhebung der murinen LOS/LPS-Resistenz [5,25,26]. Aufgrund der fehlenden IL-10-abhängigen Regulation der Immunreaktion entwickeln IL-10$^{-/-}$ Mäuse, die unter konventionellen Bedingungen aufgezogen werden, innerhalb von 9 bis 12 Wochen eine spontane chronische Kolitis als Immunantwort auf ihre eigene kommensale Darmmikrobiota. Daher wurden IL-10$^{-/-}$ Mäuse unmittelbar nach dem Absetzen vom Muttertier mit einem Antibiotika-Cocktail vorbehandelt, um die physiologische Kolonisation, die eine *C. jejuni*-Infektion verhindert, zu beseitigen und mögliche kolitogene Reize aus der Darmmikrobiota zu eliminieren, die zu einer chronischen Kolitis bei IL-10$^{-/-}$ Mäuse führen.

In diesen sekundär abiotischen IL-10$^{-/-}$ Mäusen ist *C. jejuni* nach einer peroralen Infektion in der Lage, den Darm innerhalb einer Woche in hohen Konzentrationen zu besiedeln, und die

Bakterien verursachen eine nicht-selbstlimitierende, akute Enterokolitis, die durch blutigen und entzündlichen Durchfall gekennzeichnet ist [26]. Darüber hinaus sind sowohl durch *C. jejuni* induzierte angeborene als auch adaptive pro-inflammatorische Immunantworten nicht auf den Darmtrakt beschränkt, sondern können auch in extraintestinalen Organen einschließlich systemischer Kompartimente beobachtet werden [5]. Das sekundär abiotische IL-10$^{-/-}$ Mausmodell hat sich somit für die Untersuchung der Immunopathologie der Campylobacteriose und von definierten Virulenz-Faktoren von *C. jejuni* bewährt und konnte als präklinisches Modell standardisiert werden.

1.3 Naturstoffe als neuartige immunmodulierende Therapie-Strategie bei Darmentzündungen

Pflanzen produzieren eine große Vielfalt an antimikrobiellen Molekülen, die allgemein als Phytoalexine klassifiziert werden. Die Struktur dieser Phytoalexine umfasst Terpenoide, Glykosteroide, Flavonoide und Polyphenole und ist daher sehr variabel [27]. Zahlreiche *in vitro*- und *in vivo*-Studien haben bereits die antimikrobielle Wirkung verschiedener Naturstoffe, wie zum Beispiel Resveratrol [28], Curcumin [29], Kardamom [30] und Urolithin A [31] gegen *Campylobacter* aber auch gegen diverse Viren, Pilz- und anderen Bakterienarten gezeigt. Der Einsatz dieser natürlichen Wirkstoffe gegen Pathogene könnte die Resistenzbildung gegenüber Antibiotika vermeiden; zudem, therapeutisch eingesetzt, könnten sie Infektionen verhindern und das Immunsystem stärken.

1.3.1 Ascorbinsäure (Vitamin C)

Die phenolische Verbindung Ascorbinsäure (Vitamin C) ist ein Bestandteil von Zitrusfrüchten, Kiwis, Mangos, Erdbeeren, Papayas und Tomaten, aber auch grünes Blattgemüse und Brokkoli enthalten Vitamin C in hohen Konzentrationen [32,33]. Vitamin C wird nicht nur von verschiedenen Pflanzenarten gebildet. Hefen produzieren D-Erythroascorbat, ein Ascorbat-Analogon [34]. Tiere, Pflanzen und Pilze haben sehr unterschiedliche Wege für die Vitamin-C-Biosynthese entwickelt. Bei Tieren wird D-Glucuronat, das von Uridindiphosphat-Glucuronat abgeleitet ist, zu L-Gulonat reduziert, welches in sein Lacton umgewandelt wird, das durch l-Gulonolactonoxidase zu L-Ascorbat oxidiert [35]. Interessanterweise kann die Mehrheit der Wirbeltiere Vitamin C aus Glukose synthetisieren, aber einige Säugetiere, darunter Meerschweinchen und Menschen, haben diese Fähigkeit aufgrund eines Mangels an L-Glucono-γ-Lactonoxidase verloren, die für die Synthese von Vitamin C erforderlich ist [36,37]. Daher ist eine Vitamin-C-Aufnahme durch die Nahrung zur Abdeckung des allgemeinen Bedarfs für den Menschen (100 bis 200 mg pro Tag) und zur Verhinderung von Mangelzuständen, die zu Skorbut führen, essentiell [32].

Es ist bekannt, dass Vitamin C immunstimulierende und anti-inflammatorische Eigenschaften hat, die vor Infektionen schützen können [37]. Mehrere Studien haben zudem gezeigt, dass Vitamin C antimikrobielle Wirkungen gegen verschiedene Bakterienarten wie *Mycobacterium tuberculosis* [38], *Staphylococcus aureus* [39]*, Escherichia coli* [40], *Helicobacter pylori* [41,42] und *Salmonella*-Spezies [43,44] aufweist.

Vitamin C kann *M. tuberculosis*, einschließlich mehrerer multiresistenter Stämme, direkt durch die Erzeugung oxidativer Radikale, die durch Fenton-Reaktion entstehen, abtöten [45]. In den 1980er Jahren wurde gezeigt, dass Vitamin C in Kombination mit Linalool und Kupfer synergistische Aktivitäten gegen *C. jejuni* aufweist [46-48]. Durch die Ergebnisse dieser Studien wurde auch bestätigt, dass Vitamin C (0,5 mg/mL) das Wachstum von *C. jejuni in vitro* hemmt und dass diese Wirkung durch Oxidationsprodukte wie L-Dehydroascorbinsäure oder L-Diketogulonsäure verursacht wird [46]. Es ist bemerkenswert, dass Vitamin C in Konzentrationen unter 1 mmol/L das Wachstum von *C. jejuni* stimulierte, während 5 mmol/L Vitamin C für das Pathogen letal wirkte. Diese bakterizide Wirkung wurde durch ansteigende Sterberaten von *C. jejuni* in kontaminiertem Putenfleisch nach einer Behandlung mit Vitamin C (5 mmol/kg) bestätigt [48]. Jedoch wurden bisher keine Studien durchgeführt, in denen die Wirkung von Vitamin C gegen die Campylobacteriose *in vivo* untersucht wurde.

1.3.2 Carvacrol

Das lipophile Monoterpenoid 4-Isopropyl-2-methylphenol Carvacrol ist Hauptbestandteil der ätherischen Öle von Thymian, Oregano und anderen Heilpflanzen, die eine Vielzahl von medizinisch belegten gesundheitsfördernden Wirkungen aufweisen [49]. Für Carvacrol wurde gezeigt, dass es eine Vielzahl verschiedener enzymatischer Funktionen beeinflusst, die die anxiolytischen, spasmolytischen, zellregenerativen, immunmodulierenden und tumortoxischen Aktivitäten erklären, die das Molekül im Körper des Menschen bewirkt [49,50]. Auch ist bekannt, dass die Terpenoide inklusive Carvacrol antimikrobielle Wirkungen haben, die gegen eine Vielzahl von Viren und Mikroorganismen wirksam sind.

Aktuell ist Carvacrol aufgrund seiner natürlichen antimikrobiellen Wirkung gegen mehrere durch Lebensmittel übertragene Krankheitserreger wie beispielsweise *E. coli*, *Salmonella* [51] und *Campylobacter* [52,53], im Fokus der Infektionsforschung. *In vitro*-Studien haben gezeigt, dass Carvacrol aufgrund seiner lipophilen Eigenschaften in bakteriostatischen Konzentrationen Veränderungen in der Fettsäurezusammensetzung der Bakterienzellmembranen induzieren kann [54]. In bakteriziden Konzentrationen permeabilisiert Carvacrol sogar die äußere Membran von Gram-negativen Bakterien [55]. Darüber hinaus besitzt Carvacrol eine ATPase-inhibierende Aktivität und soll als Protonenaustauscher wirken, der den pH-Gradienten über die zytoplasmatische Membran verringert. Die daraus resultierenden Änderungen des ATP-Pools wirken bakterizid und führen

zum Absterben der Bakterien [56]. Zusätzlich konnte Carvacrol die *C. jejuni*-Belastung in Darmproben von Hühnern durch die Hemmung der Expression von Virulenz-Faktoren und durch die Blockierung der *C. jejuni*-Invasion in Hühnerzellen reduzieren [57]. Daher stellt Carvacrol ein vielversprechendes Kandidatenmolekül für die Bekämpfung der humanen Campylobacteriose dar. Weitere Studien haben ebenso die anti-*C. jejuni*-Wirkung von Carvacrol in Hühnern bestätigt [57-59].

1.3.3 Cholecalciferol (Vitamin D)

Die Identifizierung von Cholecalciferol-Rezeptoren an mononukleären Zellen des peripheren Blutes in den 1980er Jahren wies erstmals auf mögliche immunmodulierende Funktionen von Vitamin D hin [60]. Der Rezeptor für Vitamin D wird von zahlreichen angeborenen und adaptiven Immunzell-Untergruppen, einschließlich Monozyten, Makrophagen, dendritischen Zellen, naiven $CD4^+$ T-Zellen, T-Helfer (Th) 1-Zellen und Th2-Zellen, exprimiert [61]. Daher ist Vitamin D sowohl an der Modulation angeborener als auch an adaptiven Immunantworten beteiligt und weist entzündungshemmende Wirkungen auf [62].

Natürliche reichhaltige Vitamin D-Quellen wie Lebertran oder Sonneneinstrahlung wurden zur Therapie der Tuberkulose eingesetzt [63]. Darüber hinaus berichten zahlreiche Studien über die antimikrobiellen Eigenschaften von Vitamin D. Das Wachstum von Gram-positiven Bakterienstämmen wie *Staphylococcus aureus*, *Streptococcus pyogenes* und *Streptococcus mutans*, aber auch von Gram-negativen Arten wie *Klebsiella pneumoniae, Helicobacter pylori* und *Escherichia coli* konnten durch den Einsatz von Vitamin D wirksam gehemmt werden [64-67]. Zusätzlich wird die Produktion von antimikrobiellen Peptiden wie Cathelicidin und Defensinen durch Vitamin D stimuliert [65].

Zahlreiche Studien haben von einer negativen Korrelation zwischen dem Vitamin D und der Aktivität entzündlicher Darmerkrankungen berichtet. Eine systematische Überprüfung und Metaanalyse von 27 Studien ergab, dass ein niedriger Vitamin D-Status mit einer erhöhten Krankheitsaktivität, Schleimhautentzündung und einem zukünftigen klinischen Rückfall sowie einer verminderten Lebensqualität bei IBD-Patienten verbunden ist [68]. Zusätzlich haben viele Studien gezeigt, dass eine Vitamin D-Behandlung die Kolitis in menschlichen und tierischen Modellen verbessert [69]. In zwei Tierstudien trug der Vitamin-D-Mangel zu einer erhöhten Schwere und Mortalität bei experimenteller Kolitis in Mäuse und sogar bei experimentellem Dickdarmkrebs bei [70,71].

Für die Behandlung der Campylobacteriose ist von Bedeutung, dass aktives Vitamin D bei der akuten Th1-induzierte Colitis die Sekretion von pro-inflammatorischen hemmt und die von entzündungshemmenden Zytokinen steigert [72], was auch zu einer beschleunigten Elimination von *C. jejuni* aus der Darmschleimhaut führen könnte [73]. Allerdings fehlen bisher

in vivo-Studien, die die anti-*C. jejuni*-Wirkung von Vitamin D nachweisen und die hiefür benötigte tägliche Vitamin D-Dosis beschreiben.

1.4 Zielsetzung

Da die Anwendung von Antibiotika bei einer Darminfektion auch die Darmmikrobiota mitunter erheblich beeinflusst und weitere Wirkstoffe gegen die Campylobacteriose fehlen, ist es von grundlegender Bedeutung, nach neuartigen antibiotika-unabhängigen Ansätzen zu suchen, um die Campylobacteriose zu behandeln und/ oder sogar zu verhindern. In unseren präklinischen Interventionsstudien haben wir die antimikrobiellen und immunmodulierenden Wirkungen von Carvacrol, Vitamin C und Vitamin D bei der Campylobacteriose im präklinischen Mausinfektionsmodel untersucht. Diese Arbeit fasst die wichtigsten Ergebnisse aus drei bereits veröffentlichten Publikationen zusammen und soll die bisher erarbeiteten Erkenntnisse im Überblick darstellen.

2. Material und Methoden

2.1 Ethische Erklärung

Die Tierversuche wurden gemäß den Europäischen Tierschutzrichtlinien (2010/63 / EU) und nach Zustimmung des Landesamtes für Gesundheit und Soziales (LaGeSo, Berlin, Registriernummern G0172/16, G0247/16 und G0104/19) durchgeführt. Der klinische Gesundheitszustand der Tiere wurde mindestens einmal täglich überprüft.

2.2 Versuchstiere und Tierhaltung

Für die vorliegende Arbeit wurden in den Räumlichkeiten der Forschungseinrichtungen für Experimentelle Medizin (FEM, Charité - Universitätsmedizin Berlin) IL-10$^{-/-}$ Mäuse (C57BL/6j Hintergrund) gezüchtet, aufgezogen und unter spezifischen pathogen-freien (SPF) Bedingungen gehalten.

Zur Eliminierung der kommensalen Darmmikrobiota wurden Mäuse im Alter von 3 bis 4 Wochen unter sterilen Bedingungen mit einer Antibiotika-Mischung behandelt. in sterile Käfige transferiert und mit einem Antibiotika-Cocktail behandelt. Dies diente der Aufhebung der physiologischen Kolonisationsresistenz, um die stabile Ansiedlung von *C. jejuni*-Kolonisation im murinen Gastrointestinaltrakt zu ermöglichen. Der Antibiotika-Cocktail bestand aus Vancomycin (500 mg/L; Cell Pharm, Hannover, Deutschland), Ciprofloxacin (200 mg/L; Bayer Vital, Leverkusen, Deutschland), Imipenem (250 mg/L; MSD, Haar, Deutschland), Metronidazol (1 g/L; Fresenius, Bad Homburg, Deutschland) und Ampicillin plus Sulbactam (1 g/L; Ratiopharm, Ulm, Deutschland). Die Antibiotika wurden in autoklaviertem Trinkwasser (*ad libitum*) gelöst und über einen Zeitraum von 8 Wochen verabreicht.

Für drei aufeinander folgende Wochen wurden Fäzesproben der Tiere in Thioglykolat-Bouillon (BD Bioscience, Heidelberg, Germany) überführt und bei 37°C inkubiert. Bei Abwesenheit kultivierbarer Bakterien wurden die Tiere als sekundär abiotisch angesehen. Um ein Auswaschen der Antibiotika sicherzustellen, wurde die Antibiotikabehandlung vier Tage vor der Infektion abgebrochen.

2.3 Behandlung mit definierten Substanzen

Umgehend nach der Unterbrechung der Antibiotika-Behandlung und somit vier Tage vor der *C. jejuni*- Infektion wurden die jeweiligen Behandlungen begonnen (Abbildung 1). Die Trinklösungen wurden alle zwei Tagen durch frisch hergestellte Lösungen ersetzt.

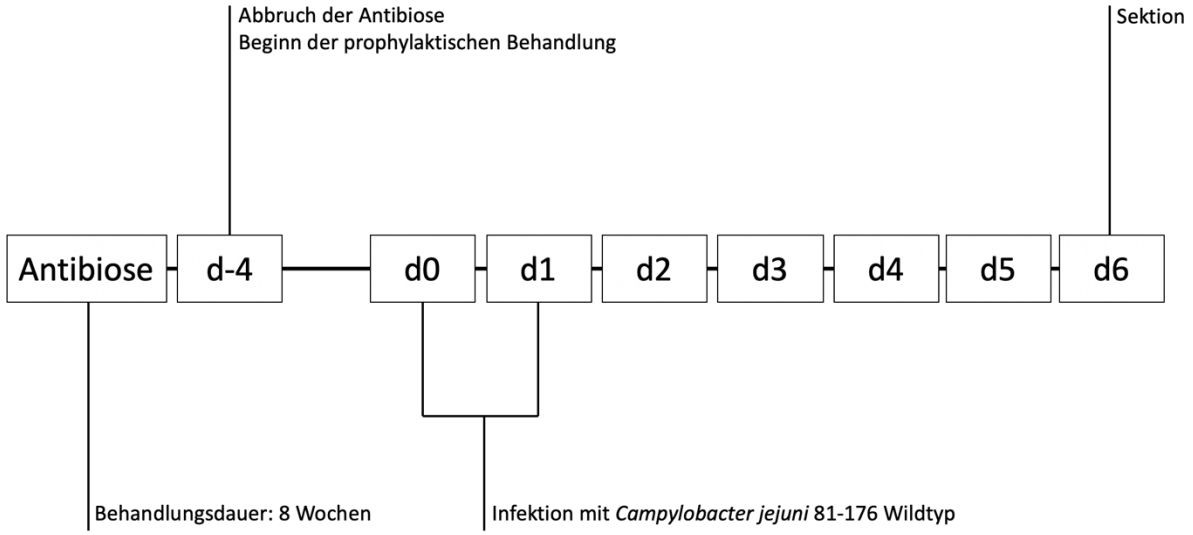

Abbildung 1: Zeitschema der durchgeführten Versuche.
Die Behandlung mit Antibiotika-Cocktail wurde vier Tage vor der Infektion (d-4) beendet und durch prophylaktische Behandlungen (Vitamin C, Carvacrol und Vitamin D) ersetzt. An zwei aufeinanderfolgenden Tagen (d0 und d1) wurden die sekundär abiotischen IL-10$^{-/-}$ Mäuse peroral mit *C. jejuni* 81-176 infiziert. Täglich wurden Fäzes-Proben zur Beurteilung der *C. jejuni*-Lasten entnommen. Der klinische Gesundheitszustand wurde ebenfalls täglich überprüft und bewertet.

Unter Berücksichtigung eines Körpergewichts von circa 25 Gramm pro Maus und eines täglichen Trinkvolumens von ca. 5 mL wurden daraus die Endkonzentrationen berechnet (Tabelle 1). Als Kontrollgruppen dienten zwei Gruppen; I) die sekundär abiotischen, unbehandelten und nicht-infizierten (naive) und II) die sekundär abiotischen, mit *C. jejuni* 81-176 infizierten Mäuse, die mit Placebo (PLC) behandelt wurden.

Tabelle 1: Dosierung der Substanzen

Name	Tägliche Dosis [pro kg Körpergewicht]	Gelöst in	End-konzentration [mg/L]	Hersteller
Ascorbinsäure* (Vitamin C)	1 g	-	5000	Sigma-Aldrich, München, Deutschland
Carvacrol**	100 mg	Tween 80 (0,2% v/v)	500	Sigma-Aldrich, München, Deutschland
25-OH-Cholecalciferol** (Vitamin D)	500 µg	Tween 80 (0,2% v/v)	2,5	Sigma-Aldrich, München, Deutschland

* Placebo (PLC)-Kontrollmäuse erhielten nur autoklaviertes Leitungswasser.
**PLC-Kontrollmäuse erhielten nur Tween 80 über das Trinkwasser.

2.4 *C. jejuni*-Infektion, -Kolonisation und -Translokation

Drei Monate alte Mäuse wurden an zwei aufeinanderfolgenden Tagen (Tag 0 und Tag 1; d0, d1) peroral infiziert. Zur Infektion wurde eine Stammlösung des *C. jejuni* 81-176-Stammes, die bei -80 ° C gelagert wurde, aufgetaut, Aliquots auf Karmali-Agar (Oxoid, Wesel, Deutschland) gestrichen und 48 Stunden bei 37°C in einer mikroaerophilen Atmosphäre inkubiert. Die perorale Infektion erfolgte mit 10^9 Kolonie-bildenden-Einheiten (KBE; eng. colony forming units, CFU) des *C. jejuni*-Stamms 81-176 in 0,3 mL Phosphat-gepufferter-Salzlösung (engl. Phosphate-buffered saline, PBS, Oxoid). Die Tiere wurden stets in einer sterilen Umgebung (autoklaviertes Futter und Trinklösung) gehalten und unter strengen aseptischen Bedingungen behandelt, um Kontaminationen zu vermeiden.

Um die Kolonisation von *C. jejuni* im Magen-Darm-Trakt zu beurteilen, wurden täglich Fäzesproben sowie am Sektionstag der luminalen Inhalt von Magen, Duodenum, Ileum und Kolon, entnommen. Die kulturelle Methoden wurden zur Bestimmung von Pathogen-Lasten verwendet.

Der Nachweis der Translokation von *C. jejuni* vom Darm zu anderen Organen erfolgte durch Kultivierung der am Tag der Sektion entnommenen Organ-Homogenate. Die jeweiligen Organ-Proben wurden in PBS homogenisiert und auf entsprechenden Agar-Nährmedien bebrütet und kulturell analysiert.. Zur Beurteilung der *C. jejuni*-Bakteriämie wurden Thioglykolat-Bouillons mit 200 µL Herzblut einzelner Mäuse inokuliert, 7 Tage bei 37°C inkubiert und zur weiteren Identifizierung auf Festmedien kultiviert.

2.5 Probengewinnung

Die Mäuse wurden am Tag 6 nach der Infektion mittels Isofluran-Inhalation (Abbott, Deutschland) getötet. Zusätzlich zu den Proben des luminalen Inhaltes wurden *ex vivo* Biopsien aus Kolon, Ileum, mesenterialen Lymphknoten (engl. mesenteric lymph nodes, MLN),

Milz, Leber, Nieren und Lunge unter sterilen Bedingungen entnommen. Zur Bestimmung der Zytokin-Konzentrationen in Serum wurde Herzblut entnommen. Kolon- und extraintestinale Proben wurden von jeder Maus parallel für mikrobiologische, immunhistopathologische und immunologische Analysen entnommen. Die absoluten Kolon- und Ileumlängen wurden mit einem Lineal gemessen.

2.6 Immunhistochemie

Für die *In-situ*-immunhistochemische Analysen wurden sowohl die extraintestinalen *ex vivo*-Biopsien als auch Proben des Darms in 5%- igem Formalin fixiert, in Paraffin eingebettet und in 5 µm dünne Schichten geschnitten und nach anschließendem Entparaffinieren (mittels Xylols, 96%-igem und 70%-igem Ethanol) mit spezifischen Antikörpern gefärbt.
Verwendet wurden primäre Antikörper gegen gespaltene Caspase-3 für apoptotische Zellen (Asp175, Cell Signaling, Beverly, MA, USA, 1:200), Ki67 für proliferierende Zellen (TEC3, Dako, Glostrup, Dänemark, 1:100), CD3 für T-Lymphozyten (Nr. N1580, Dako, 1:10) FOXP3 für regulatorische T-Zellen (clone FJK-165, Nr. 14-5773, eBioscience, San Diego, CA, USA, 1:100), B220 für B-Lymphozyten (Nr. 14-0452-81, eBioscience, 1:200) und F4/80 für Monozyten/Makrophagen (Nr. 14-4801, clone BM8, eBioscience, CA, USA, 1:50).

Die Schnitte wurden 30 Minuten mit den primären Antikörpern inkubiert, gefolgt von weiteren 30 Minuten Inkubation mit den jeweiligen sekundären Antikörpern (für Anti-Caspase-3- und Anti-CD3-Färbung: biotinylierter donkey anti-rabbit-Antikörper; für anti-F4/80, anti-Ki67 und Anti-B220: biotinylierter rabbit anti-rat-Antikörper; für anti-FOXP3 biotinylierter donkey anti-rat-Antikörper; alle aus Dianova, Hamburg, Deutschland). Als Detektionssystem wurde das Dako REAL™ Streptavidin-Alkaline Phosphatase/RED Rabbit/Mause (Agilent, Santa Clara, CA, USA) Kit verwendet. Bei den negativen Kontrollen wurden die primären Antikörper ausgeschlossen.

Um die unspezifischen Bindungsstellen zu blockieren, wurde jeder Gewebeschnitt mit 150 µL Dako Dual Endogenic Enzyme Block für 10 Minuten inkubiert und anschließend mittels TBST-Puffer (eng. Tris-buffered saline with Tween20, TBST) gespült. Danach wurde der primäre Antikörper in Dako REAL™ Antikörperverdünnungsmittel verdünnt und 150 µL der Lösung auf jeden Objektträger aufgetragen. Nach 30 Minuten Inkubation wurden ungebundene Antikörper mit TBST gespült. Der sekundäre Antikörper wurde auch in Dako REAL™ Antikörperverdünnungsmittel verdünnt und 150 µL der Lösung wurden 30 Minuten lang auf jeden Gewebeschnitt aufgetragen. Nach dem Spülen der ungebundenen Antikörper mit TBST wurden 2 Tropfen Dako REAL™ Streptavidin Alkaline Phosphatase mit einer Inkubationsdauer von 30 Minuten auf jeden Gewebeschnitt aufgetragen. Die ungebundene alkalische Phosphatase wurde mit TBST gespült und die Entwicklerlösung hergestellt. Dafür

wurde 1 Tropfen Dako REAL™ Levamisol zu jedem 10 mL Dako REAL™ AP-Substratpuffer gegeben, um die endogene alkalische Phosphatase zu blockieren. Anschließend wurden die Dako REAL™ Chromogen Red Compounds zugegeben (jeweils 30 µL pro 750 µL Substratpuffer). 200 µL der Entwicklerlösung wurden auf jeden Gewebeschnitt aufgetragen und 30 Minuten inkubiert. Anschließend wurden die Objektträger mit PBS gespült.
Unmittelbar nach der Immunreaktion wurden die Objektträger mit destilliertem Wasser gewaschen und die Gewebeschnitte mit Mayer-Hemalum-Lösung gefärbt, um alle basophilen Zellstrukturen (z.B. DNA) zur besseren Beurteilung der immunhistoschemischen Ergebnisse darzustellen. Nach einer Inkubationsdauer von 20 Sekunden wurden die Objektträger gründlich mit Leitungswasser gewaschen, was zu einer pH-Verschiebung führt, die zu einer Änderung der Farbe führt (rot nach blau).
Mittels Lichtmikroskopie wurde die durchschnittliche Anzahl der jeweiligen positiv-gefärbten Zellen *in situ* innerhalb von mindestens sechs hochauflösenden Feldern (engl. high power fields, HPF; 0,287 mm², 400-fache Vergrößerung) von einem unabhängigen Untersucher ermittelt.. Zur Erzeugung von Bildern wurde ein Axiolmager Z1-Mikroskop verwendet, welche anschließend mit der Axiovision-Software (Carl Zeiss MicroImaging, Jena, Deutschland) verarbeitet wurden.

2.7 Zytokin-Messungen in Überständen von *ex vivo*-Biopsien aus Kolon und extraintestinalen Organen

Kolon- und Ileumgewebe (jeweils längs aufgeschnittener Streifen von ca. 1 cm^2) sowie *ex vivo*-Biopsien aus MLN (3 - 4 Lymphknoten), Milz (eine Hälfte), Niere (eine Hälfte nach Längsschnitt), Leber (ca. 1 cm^3) und Lunge wurden in sterilem PBS ausgewaschen und für 18h bei 37°C auf Kulturplatten mit 24-fachem Boden (Nunc, Deutschland), die 500µl serumfreies RPMI 1640-Medium (Gibco, Life Technologies, UK), Penicillin (100 U/mL) und Streptomycin (100 µg / ml; PAA Laboratories, Deutschland) enthielt, kultiviert.
Im Anschluss wurden die Kulturüberstände und Serumproben auf die Mediatoren Monocyte Chemoattractant Protein (MCP)-1, Interferon (IFN) -γ, Tumornekrosefaktor (TNF) und IL-6 mittels „Inflammation Cytometric Bead Array“ (CBA; BD Biosciences) und FACS Canto II (BD Biosciences) quantifiziert. Das Stickstoffmonoxid (NO) -Konzentrationen wurden mittels Griess-Reaktion bestimmt.

2.8 Statistische Analysen

Für jede Behandlung wurden Daten von vier unabhängig durchgeführten Experimenten gepoolt und analysiert. Der Mann-Whitney-Test (Prism, Version 7, GraphPad, San Diego, USA) wurde zur Bestimmung von Medianen und Signifikanzniveaus für paarweise Vergleiche

nicht normalverteilter Daten verwendet, während für Mehrfachvergleiche der einseitige ANOVA-Test mit Tukey-Nachkorrektur oder der Kruskal-Wallis-Test mit Dunn‘s-Nachkorrektur verwendet. Zweiseitige Wahrscheinlichkeitswerte $p \leq 0{,}05$ wurden als signifikant angesehen.

3. Ergebnisse

Zur Darstellung der Resultate werden Auszüge der Ergebnisse der drei hier zusammengefassten Publikationen in ihrer Reihenfolge (Publikation 1: Vitamin C, 2: Carvacrol, und 3: Vitamin D) präsentiert. Auf die jeweilige graphische Darstellung der Ergebnisse wird entsprechend auf die Abbildungsnummer der Originalpublikation hingewiesen.

3.1 Auszüge der Untersuchungsergebnisse zur Auswirkung der prophylaktischen Vitamin C-Behandlung in *C. jejuni*-infizierten sekundär abiotischen IL-10$^{-/-}$ Mäusen (Publikation 1)

Die durch Antibiotika-Behandlung generierten sekundär abiotischen IL-10$^{-/-}$ Mäuse wurden vier Tage vor der *C. jejuni*-Infektion (d-4) mit synthetischem Vitamin C über das Trinkwasser (5 g/L) behandelt. Die Vitamin C-Behandlung wurde bis zum Sektionstag (d6) fortgeführt. An den Tagen 0 und 1 wurden die Mäuse peroral mit 10^9 lebensfähigen *C. jejuni*-Bakterien infiziert. Die täglichen Bakterienkulturanalysen von Stuhlproben ergaben, dass eine Vitamin C-Behandlung im Vergleich zur PLC-Behandlung die Darmbesiedlungseigenschaften des verwendeten *C. jejuni*-Stammes nicht beeinflusst (Publikation 1; Abb. S2). Am Tag 6 (d6) waren jedoch die *C. jejuni*-Lasten im Kolon im Vergleich zu mit PLC behandelten Mäusen geringfügig niedriger. Die Pathogen-Dichten des Magen-Darm-Traktes einschließlich Magen, Duodenum und Ileum waren zwischen Vitamin C- und PLC-behandelten Mäusen vergleichbar (Publikation 1; $p<0.001$; Abb. 1). Daher wird die intestinale *C. jejuni*-Kolonisierung durch die Vitamin C-Behandlung nur begrenzt beeinflusst.

Es wurde weiterhin eine tägliche Untersuchung der *C. jejuni*-induzierten Symptome bei infizierten Mäusen durchgeführt, wobei ein standardisiertes klinisches Bewertungssystem angewendet wurde, bei dem das Erscheinungsbild von Mäusen, die Stuhlkonsistenz und die Häufigkeit vom Blut in Stuhlproben bewertet wurden. Die Untersuchung des klinischen Bildes im zeitlichen Verlauf ergab, dass die mit Vitamin C behandelten Tiere im Vergleich zu den mit PLC behandelten Tieren bereits am Tag 5 nach der Infektion deutlich weniger Symptome der *C. jejuni*-Infektion zeigten (Publikation 1; Abb. S3). Am Tag 6 nach der Infektion litten mit PLC behandelte Mäuse an Infektionssymptomen und schwerem blutigen Durchfall, was auf eine akute Enterokolitis hinweist, während die Behandlung mit Vitamin C zu einer signifikanten Verringerung der durch *C. jejuni* induzierten Symptome führte (Publikation 1; $p<0{,}05–0{,}001$;

Abb. 2). Bemerkenswerterweise zeigten 40% der infizierten Mäuse, die mit Vitamin C behandelt worden waren, makroskopisch keine Symptome der Infektion (Publikation 1; p<0,001; Abb. 2A; Abb. S3). Somit vermindert eine Behandlung mit Vitamin C die klinischen Symptome der Campylobacteriose.

Für die Analyse der durch die *C. jejuni*-Infektion induzierte Darmentzündung, wurde die Anzahl der apoptotischen Kolonepithelzellen nach immunhistochemischer Färbung von Kolonparaffinschnitten mit einem Antikörper gegen die gespaltene Caspase 3 quantitativ bestimmt. Im Vergleich zu naiven Tieren war die *C. jejuni*-Infektion mit einem vielfachen Anstieg der apoptotischen Epithelzellen des Kolons assoziiert (Publikation 1; p<0,001; Abb. 3B), während dieser Anstieg bei Vitamin C-behandelten Mäusen weitaus weniger ausgeprägt war (Publikation 1; p<0,001; Abb. 3B; Abb. S4). Weiterhin verursachte die *C. jejuni*-Infektion einen deutlichen Anstieg von Immunzellen der angeborenen Immunität wie etwa Makrophagen und Monozyten (Publikation 1; p<0,001; Abb. 4A; Abb. S5a), aber auch von adaptiven Immunzellen einschließlich T- und B-Lymphozyten (Publikation 1; p<0,01–0,001; Abb. 4B und C; Abb. S5b und c). Im Vergleich zu PLC-Kontrolltieren zeigten Mäuse, die mit Vitamin C behandelt worden waren, eine geringere Anzahl von Makrophagen/Monozyten sowie von T- und B-Lymphozyten in ihrer Dickdarmschleimhaut und Lamina propria (Publikation 1; p<0,01–0,001; Abb. 4; Abb. S5). Somit vermindert die Vitamin C-Behandlung die durch *C. jejuni* induzierte Apoptose und die Immunzellreaktionen im Dickdarm.

Ergänzend wurden pro-inflammatorische Mediatoren im Darm-Explantaten untersucht. Während die mit PLC behandelten Mäusen erhöhte NO- und TNF-Konzentrationen in *ex vivo* Biopsien des Dickdarms zeigten (Publikation 1; p<0,01–0,001; Abb. 5A und B), wiesen die mit Vitamin C behandelten Mäusen niedrigere Mediatorwerte in ihrem Dickdarm auf (Publikation 1; p<0,05; Abb. 5A und B).

Im Einklang mit der erhöhten Anzahl an Immunzellen zeigten die PLC-behandelten Mäuse im Vergleich zu den mit Vitamin C behandelten Tieren ebenfalls erhöhte IL-6- und IFN-γ-Konzentrationen in Kolon-Biopsien (Publikation 1; Abb. 5C und D). Dies traf auch für IFN-γ-Spiegel in MLN zu (Publikation 1; p <0,001; Abb. 5E). Demnach dämpft die Behandlung mit Vitamin C die Sekretion pro-inflammatorischer Mediatoren v.a. im Dickdarm *C. jejuni*-infizierter Mäuse.

Die Wirkungen von Vitamin C wurden ebenfalls in extraintestinalen Organen untersucht. Die quantitativen Bewertungen der immunhistochemischen Untersuchen in Nieren, Lunge und Leber zeigten eine erhöhte Anzahl an apoptotische Zellen nach der *C. jejuni*-Infektion in den Nieren und Lungen der PLC-Gruppe, jedoch nicht in den Organen der Vitamin C behandelten Mäuse (Publikation 1; Abb. 6A, B; Abb. S6a, b).

Im Vergleich zu naiven Tieren wiesen beide infizierten Behandlungsgruppen erhöhte apoptotische Zellzahlen in der Leber auf (Publikation 1; p<0,01–0,001 vs. naiv), während diese

in der Vitamin C-behandelten Gruppe weniger stark ausgeprägt war (Publikation 1; Abb. 6C; Abb. S6c). Interessanterweise zeigten sich nur bei den PLC-behandelten Mäusen eine erhöhte Anzahl an T-Lymphozyten (Publikation 1; Abb. 7A; Abb. S6d) sowie eine verstärkte TNF-Sekretion (Publikation 1; $p<0.05$; Abb. 7B) in der Leber im Vergleich zu naiven Tieren. Diese Ergebnisse weisen auf die krankheitsmindernden Wirkungen der Vitamin C-Behandlung während einer *C. jejuni*-Infektion hin, die nicht nur auf den Darmtrakt beschränkt war, sondern auch in extraintestinalen Kompartimenten beobachtet werden konnte.

3.2 Auszüge der Untersuchungsergebnisse zur Auswirkung der prophylaktischen Carvacrol-Behandlung von *C. jejuni*-infizierten, sekundär abiotischen IL-10$^{-/-}$ Mäusen (Publikation 2)

Die gesundheitsfördernden Eigenschaften von synthetischem Carvacrol (500 mg/L) während der *C. jejuni*-Infektion wurden in demselben Mausmodell und mit einem identischen Versuchsdesign (wie in 3.1 beschrieben) untersucht.

Am Tag 6 nach der Infektion wiesen PLC-behandelte Kontrollmäuse im Kolon und im Ileum Pathogen-Lasten von 10^8 bzw. 10^9 CFU/g auf, die bei den Carvacrol-behandelten Mäusen im Vergleich zu den PLC-Kontrollen um etwa 0,5 bzw. 2,0 logarithmische Stufen niedriger ausfielen (Publikation 2; $p<0,01$ bzw. $p<0,001$; Abbildung. 1). Folglich senkt die Carvacrol-Behandlung die *C. jejuni*-Dichten im Darm um bis zu 2 Größenordnungen.

Im Vergleich zur PLC-Gruppe, die an einer akuten Enterokolitis litt, zeigten mit Carvacrol-behandelten Mäuse bereits 48 Stunden nach der *C. jejuni*-Infektion deutlich weniger Symptome der Campylobacteriose. Diese Wirkung von Carvacrol war bis zum Sektionstag (d6) zu beobachten (Publikation 2; $p<0,001$ Abb. 2; Abb. S1). Bemerkenswerterweise litten alle PLC-behandelten Mäuse an Diarrhoe, während nur 10,5% der mit Carvacrol behandelten Tiere davon betroffen waren (Publikation 2; $p<0,001$; Abb. 2c). Somit konnten die *C. jejuni*-induzierten Symptome durch die Carvacrol-Behandlung gebessert werden.

Als nächstes untersuchten wir, ob die positiven Wirkungen der Carvacrol-Behandlung auf der makroskopischen Ebene ebenso auf mikroskopischer Ebene beobachtet werden konnten. Hierfür wurden paraffinfixierte Kolonschnitte für eine *in situ* immunhistochemische Untersuchung mit definierten Antikörpern gegen verschiedene entzündliche und proliferative/regenerative Zellmarker sowie gegen verschiedene Immunzellpopulationen gefärbt.

Im Vergleich zu naiven Tieren zeigten sowohl PLC- als auch Carvacrol-behandelte Mäuse eine erhöhte Anzahl von Caspase3$^+$ apoptotischen Zellen in ihren Kolonepithelien (Publikation 2; $p<0,001$ vs. naiv; Abb. 3a). Jedoch war die Anzahl apoptotischer Zellen in Kolonepithelien von

Carvacrol im Vergleich zu PLC-behandelten Mäusen signifikant niedriger (Publikation 2; p <0,001; Abb. 3a; Zusatzdatei 2: Abb. S2A).

Als nächstes wurden die paraffinfixierte Kolonschnitte mit Antikörpern gegen Ki67 gefärbt, wodurch die Zellproliferation und -regeneration untersucht werden sollte. Tatsächlich verursachte die *C. jejuni*-Infektion einen deutlichen Anstieg der Ki67-positiven Kolonepithelzellen (Publikation 2; p<0,001 vs. naiv; Abb. 3b; Zusatzdatei 2: Abb. S2B). Interessanterweise war die Anzahl der proliferativen/regenerativen Zellen in Carvacrol- im Vergleich zu PLC-behandelten Mäusen deutlich höher (Publikation 2; p<0,001; Abb. 3b; Zusatzdatei 2: Abb. S2B). Demnach verbesserte die Carvacrol-Behandlung die murine Campylobacteriose, indem sie die Apoptose hemmte und die Regenerationsprozesse in den Kolonepithelien stimulierte.

Als nächstes wurde die Immunantworten im Kolon von *C. jejuni*-infizierten Mäusen nach der Carvacrol-Behandlung untersucht. Verschiedene Immunzellpopulationen wie $CD3^+$ T-Lymphozyten und $B220^+$ B-Lymphozyten stiegen in der Kolonschleimhaut und der Lamina propria von Mäusen aus beiden Behandlungsgruppen um ein Vielfaches im Vergleich zu naiven Tieren an (Publikation 2; p<0,001; Abb. 3c und d; Zusätzliche Datei 2: Abb. S2C, D). Auch der Anstieg an T-Lymphozyten im Dickdarm der Carvacrol-behandelten Mäuse war im Vergleich zur PLC-Gruppe signifikant geringer (Publikation 2; p<0,005, Abb. 3c; Zusatzdatei 2: Abb. S2C). Zusätzlich zeigten beide infizierten Experimentalgruppen eine verstärkte Sekretion der pro-inflammatorischen Mediatoren NO, TNF und IL-6, die in *ex vivo*-Darmbiopsien gemessen wurde (Publikation 2; p<0,05–0,001; Abb. 4a, c, d). Im Vergleich zur PLC-Anwendung führte die Carvacrol-Behandlung jedoch zu signifikant niedrigeren NO-, IFN-γ- und TNF-Konzentrationen im Dickdarm (Publikation 2; p<0,05–0,001; Abb. 4a - c). Bemerkenswerterweise gab es keinen Unterschied zwischen dem IFN-γ-Spiegeln der Carvacrol-behandelten Mäuse und den naiven Kontrollmäusen zu verzeichnen (Publikation 2; n.s.; Abb. 4b). Folglich hemmte die Carvacrol-Behandlung die *C. jejuni*-induzierte Entzündung im Kolon.

Wie im Kolon beobachtet, waren erhöhte Konzentrationen von pro-inflammatorischen Mediatoren wie IFN-γ, TNF und IL-6 in MLN nur bei den PLC-behandelten Mäusen messbar, während zwischen den naiven und Carvacrol-behandelten Mäusen es keinen signifikanten Unterschied gab (Publikation 2; Abb. 5).

Wir untersuchten weiterhin die krankheitsmindernden Eigenschaften der Carvacrol-Behandlung in extraintestinalen einschließlich systemischer Kompartimente. Eine erhöhte Anzahl von $Caspase3^+$ apoptotischen Zellen konnte in Paraffinschnitten aus beiden infizierten Gruppen (PLC und Carvacrol) beobachtet werden, die am Sektionstag (d6) aus Leber, Nieren und Lunge entnommen wurden, während die Anzahl der apoptotischen Zellen in Nieren und Lungen von Carvacrol-behandelten Mäusen im Vergleich zu den PLC-Kontrollen signifikant

niedriger ausfiel (Publikation 2; Abb. 6; Zusatzdatei 6: Abb. S6). Bemerkenswerterweise unterschied sich die Anzahl der apoptotischen Zellen in der Lunge von Carvacrol-behandelten *C. jejuni*-infizierten und naiven Mäusen nicht voneinander (Publikation 2; n.s.; Abb. 6c, zusätzliche Datei 6: Abb. S6C).

Als nächstes wurde die Sekretion der pro-inflammatorischen Zytokinen in jeweiligen *ex vivo* Biopsien gemessen. Am Tag 6 nach der Infektion wurden im Vergleich zu PLC-behandelten Mäusen niedrigere IFN-γ- und TNF-Konzentrationen in der Leber von Carvacrol-behandelten Mäusen gemessen (Publikation 2; $p<0,05$ bzw. $p<0,001$; Abb. 7a, b). Dies gilt ebenfalls für die in den Nieren gemessenen IFN-γ-Konzentrationen ($p<0,05$; Fig. 7c), jedoch nicht für die renalen TNF-Werte (Publikation 2; n.s.; Abb. 7d). Außerdem wurden nach einer *C. jejuni*-Infektion erhöhte IFN-γ Konzentrationen in den Lungen von PLC- (Publikation 2; $p<0,05$; Abb. 7e), jedoch nicht von Carvacrol-behandelten Mäusen (Publikation 2; n.s.; Abb. 7e) gemessen. Unabhängig vom Behandlungsschema waren erhöhte pulmonale TNF-Konzentrationen zu verzeichnen ($p<0,05$; Fig. 7f).

Bemerkenswerterweise konnten die entzündungshemmenden Wirkungen von Carvacrol auch systemisch (d.h. im Serum) beobachtet werden. Im Vergleich zu PLC-behandelten Mäusen zeigte die Carvacrol-Gruppe deutlich geringere Serum-Konzentrationen von pro-inflammatorischen Mediatoren wie IFN-γ, TNF, MCP-1 und IL-6 (Publikation 2; $p<0,05$–$0,005$; Abb. 8). Somit dämpft die Carvacrol-Behandlung die Folgen der *C. jejuni*-Infektion nicht nur in intestinalen, sondern auch in extraintestinalen und sogar in systemischen Kompartimenten.

3.3 Auszüge der Untersuchungsergebnisse zur Auswirkung der prophylaktischen Vitamin D-Behandlung in *C. jejuni*-infizierten, sekundär abiotischen IL-10$^{-/-}$ Mäusen (Publikation 3)

Sekundär abiotische IL-10$^{-/-}$ Mäuse wurden 4 Tage vor der *C. jejuni*-Infektion mit synthetischem 25-OH-Cholecalciferol (Vitamin D) über das Trinkwasser prophylaktisch behandelt. An zwei aufeinanderfolgenden Tagen, nämlich an den Tagen 0 und 1, wurden die Mäuse dann peroral mit 10^9 lebensfähigen *C. jejuni*-Bakterien infiziert. Tägliche kulturelle Analysen von Stuhlproben nach der Infektion und am Tag der Autopsie ergaben, dass im Vergleich zur PLC-Gruppe die Vitamin D-Anwendung keinen Einfluss auf die Besiedlung von Magen, Duodenum, Ileum und Kolon durch *C. jejuni* hatte (Publikation 3; n.s.; Abb. 1, S1). Folglich hatte die Behandlung mit synthetischem Vitamin D keinen Einfluss auf die *C. jejuni*-Lasten im Gastrointestinaltrakt.

Innerhalb der ersten 6 Tage nach der *C. jejuni*-Infektion entwickelten Mäuse aus beiden Behandlungsgruppen vergleichsweise schwere Symptome einer akuten Enterokolitis, die täglich unter Verwendung eines standardisierten kumulativen klinischen Bewertungssystems (Publikation 3; Abb. S2) quantifiziert wurden. Während die durch das Pathogen induzierten

klinischen Symptome in den beiden Behandlungsgruppen im zeitlichen Verlauf vergleichbar waren (Publikation 3; n.s.; Abb. S2), litten die Vitamin D-behandelten Mäusen im Vergleich zur PLC-Gruppe bereits 24 Stunden nach der letzten Infektion (d2) weniger an Durchfall (Publikation 3; Abb. 2). Dies wurde auch an beiden darauffolgenden Tagen (d.h. d3 und d4) beobachtet. Somit führte die Vitamin D-Behandlung zu einer weniger ausgeprägten *C. jejuni*-induzierten Durchfallsymptomatik.

Da die Apoptose einen verlässlichen Parameter für die Beurteilung des Schweregrades der entzündlichen Gewenbsveränderungen im Rahmen einer *C. jejuni*-Infektion darstellt, wurden Caspase3$^+$ apoptotische Epithelzellen in Kolon-Biopsien mittels *in situ* Immunhistochemie quantitativ untersucht. Am Tag 6 nach der Infektion zeigten die *C. jejuni*-infizierten Mäuse eine vielfach erhöhte Anzahl apoptotischer Zellen in ihren Kolonepithelien, die im Vergleich zu Vitamin D-behandelten Mäusen um mehr als 60% höher lag (Publikation 3; $p<0,05$; Abb. 3A, Abb. S4A). Umgekehrt zeigten die Vitamin D-behandelten Mäuse eine deutlich höhere Anzahl der Ki67$^+$ Kolonepithelzellen im Vergleich zur PLC-Behandlung (Publikation 3; $p<0,05$; Abb. 3B, S4B). Demnach hemmt eine Vitamin D-Behandlung die *C. jejuni*-induzierten apoptotischen Veränderungen im Kolonepithel und fördert zellregenerative Maßnahmen, die einer Schädigung der Darmzellen entgegenwirken.

Im weiteren Verlauf wurden sowohl die angeborene als auch die adaptive Immunzellreaktionen im Kolon mittels immunhistochemischer Färbung von Kolonparaffinschnitten untersucht. Durch die *C. jejuni*-Infektion stieg die Anzahl der innaten F4/80$^+$ Makrophagen und Monozyten (Publikation 3; Abb. 4A, S4C) sowie adaptiver Immunzellen wie CD3$^+$ Lymphozyten (Publikation 3; Abb. 4B, S4D) in der Kolonschleimhaut und der Lamina propria an, jedoch fiel dieser Anstieg bei Vitamin D-behandelten Mäusen im Vergleich zur PLC-Gruppe geringer aus. Interessanterweise war die Anzahl der FOXP3$^+$ regulatorischen T-Zellen nach Vitamin D-Behandlung im Vergleich zur PLC-Behandlung geringfügig höher (Publikation 3; $p<0,01$; Abb. 4C, S4E). Somit führt die Vitamin D-Behandlung zu einer weniger stark ausgeprägten *C. jejuni*-induzierten Akkumulation bestimmter Zellpopulationen der angeboren und adaptiven Immunität.

Anschließend wurden die Konzentrationen pro-inflammatorischer Mediatoren in *ex vivo* Biopsien des Darmes gemessen. Die IL-6- und MCP-1-Konzentrationen stiegen nach der *C. jejuni*-Infektion im Kolon von PLC-behandelten Mäusen, jedoch nicht bei Vitamin D-behandelten Mäusen an (Publikation 3; Abb. 5A, B). Jedoch wurden die *C. jejuni*-induzierten erhöhten TNF- und IFN-γ-Konzentrationen im Kolon nicht von der Vitamin D-Gabe beeinflusst (Publikation 3; Abb. 5C, D). Somit vermindert eine Vitamin D-Behandlung entzündungsfördernde Mediatoren im Darmtrakt im Zuge einer *C. jejuni*-Infektion.

Des Weiteren wurden die entzündungshemmenden Wirkungen von Vitamin D ebenso auf extraintestinaler Ebene untersucht. Tatsächlich waren die IFN-γ-Konzentrationen in MLN und

Leber von Vitamin D-behandelten Mäusen im Vergleich zur PLC-Gruppe deutlich geringer (Publikation 3; $p<0,05$; Abb. 7A, B). Interessanterweise führte die *C. jejuni*-Infektion, unabhängig vom Behandlungsschema, zu einer verminderten IFN-γ-Sekretion in *ex vivo* Biopsien der Milz (Publikation 3; $p <0,001$; Abb. 7C). Somit führte die Vitamin D-Behandlung zu einer niedrigeren *C. jejuni*-induzierten IFN-γ-Sekretion in MLN und Leber.
Um der Frage nachzugehen, ob eine Vitamin D-Behandlung auch die systemischen pro-inflammatorischen Immunantworten, die durch *C. jejuni* induziert werden, lindern kann, wurden bestimmte Entzündfungsmediatoren im Serum gemessen. Sechs Tage nach der Infektion zeigten Mäuse aus beiden Behandlungsgruppen vergleichbar erhöhte IL-6-, MCP1-, TNF- und IFN-γ-Serumkonzentrationen (Publikation 3; Abb. S5). Somit werden die nach einer *C. jejuni*-Infektion erhöhten systemischen pro-inflammatorischen Mediatoren durch eine Vitamin D-Behandlung nicht beeinflusst.

4. Diskussion

In diesen präklinischen Interventionsstudien wurde erstmals die potenziellen antipathogenen und immun-modulierenden Eigenschaften von Vitamin C, Carvacrol und Vitamin D während der akuten Campylobacteriose in einem klinischen Mausinfektionsmodell untersucht.

4.1 Auswirkung der Vitamin C-Vorbehandlung in *C. jejuni*-infizierten, sekundär abiotischen IL-10$^{-/-}$ Mäusen

Da Vitamin C wasserlöslich ist und die Konzentrationen, die den täglichen Bedarf überschreiten, über die Nieren ausgeschieden werden, ist das Risiko von Nebenwirkungen und Vergiftungen bei hochdosierter Vitamin C Einnahme bei intakter Nierenfunktion als eher gering einzustufen [32]. Daher kommt hoch dosiertes Vitamin C als Therapiemöglichkeit gegen die Campylobacteriose in Frage, auch wenn eine Diarrhoe als Nebenwirkung gegebenenfalls in Kauf genommen werden muss. Mit unserer präklinischen Interventionsstudie wurden die antibakteriellen und immunmodulierenden Eigenschaften von hoch dosiertem Vitamin C in einem murinen Infektionsmodell nachgewiesen.
Die *C. jejuni*-Infektion in Vitamin C-vorbehandelten sekundär-abiotischen IL-10$^{-/-}$ Mäusen resultierte in einer stabilen *C. jejuni*-Kolonisation und entsprechend hohen intestinalen Pathogen-Lasten (bis zu 10^9 CFU/g Fäzes), welche bis zum sechsten Tag nur geringfügig um weniger als eine Größenordnung gesenkt werden konnte. Die antibakteriellen Eigenschaften von Vitamin C gegen durch Lebensmittel übertragbare Krankheitserreger wie *C. jejuni* und *Salmonella* wurden durch mehrere frühere *in vitro* Studien bestätigt. Die pH-senkende Eigenschaften von Vitamin C erklärten dessen antimikrobielle Wirksamkeit [74]. Dies ist jedoch

nicht der Fall, was die antimikrobielle Aktivität von Vitamin C gegen *C. jejuni* betrifft, die eher von der Oxidation von Vitamin C zu Dehydroascorbinsäure und anderen Produkten abhängt [32,33].

Trotz der hohen Pathogenlasten im Darm führte die Vitamin C-Behandlung zur besseren klinischen Symptomatik in *C. jejuni*-infizierten Mäusen, die im Vergleich zur PLC-Gruppe weniger an schwerem Durchfall und weniger blutigem Stuhl litten. Diese krankheitslindernde Wirkung von Vitamin C ging mit weniger stark ausgeprägten apoptotischen Kolonepithelzellreaktionen (im Vergleich zu PLC-Behandelten Tieren) bestätigt. Unsere Befunden werden zudem durch eine mittels Vitamin C verminderte *Helicobacter pylori*-induzierte Apoptose in menschlichen Magenepithelzellen [75] sowie reduzierte *E. coli*-induzierte Apoptose in humanen Neutrophilen [76] und die Unterdrückung der apoptotischen Signalwege in menschlichen Monozyten aufgrund der intrazellulären Vitamin C-Akkumulation [77] unterstützt.

Zusätzlich führte die Vitamin C-Behandlung zu weniger stark ausgeprägten *C. jejuni*-induzierten pro-inflammatorischen Zelleaktionen des angeborenen sowie adaptiven Immunsystems, die durch eine geringere Anzahl von Makrophagen und Monozyten sowie von T- und B-Lymphozyten im entzündeten Dickdarm gekennzeichnet war. Infolgedessen resultierte eine geringere Sekretion von pro-inflammatorischen Mediatoren wie etwa NO, TNF und IFN-γ und IL-6 innerhalb der entzündeten Kolonschleimhaut.

Frühere Studien haben gezeigt, dass die Inkubation von LPS-stimuliertem menschlichem Blut mit Vitamin C mit verminderten TNF-Spiegel einherging [78]. Ebenso reduzierte Vitamin C die TNF-Spiegel in T-Zellkulturen, die aus murinen Milzen gewonnen waren [79]. Außerdem führte eine Kombinationsbehandlung von Vitamin C und Antibiotika zu verringerten Konzentrationen von NO, TNF, IFN-γ und IL-6 in Peritoneal-Makrophagen, die aus *Staphylococcus aureus*-infizierten Mäusen isoliert wurden [80].

Interessanterweise waren die entzündungshemmenden Wirkungen der Vitamin C-Behandlung bei *C. jejuni*-infizierten Mäusen nicht nur auf den Darmtrakt beschränkt, sondern konnten auch in extraintestinalen Organen beobachtet werden. Im Vergleich zu PLC-behandelten Mäuse zeigten die Vitamin C-behandelten Tiere weniger stark ausgeprägte apoptotische Zellveränderungen in Leber, Nieren und Lunge, niedrigere T-Zellen-Zahlen sowie verminderte TNF-Konzentrationen in Lebern.

Zusammenfassend liefert unsere präklinische Interventionsstudie zum ersten Mal Hinweise, dass die prophylaktische Applikation von Vitamin C als Nahrungsergänzungsmittel eine Therapie bei akuter Campylobacteriose ermöglicht. Dieser Effekt ist auf die starke entzündungshemmende, Abwehrmechanismen-verstärkende und in der Konsequenz krankheitslindernde Wirkung von Vitamin C zurückzuführen.

4.2 Auswirkung der Carvacrol-Vorbehandlung in *C. jejuni*-infizierten, sekundär abiotischen IL-10$^{-/-}$ Mäusen

Die mit *C. jejuni* infizierten, sekundär abiotischen IL-10$^{-/-}$ Mäuse weisen klinischen Merkmale einer schweren Campylobacteriose auf [26]. Daher wurde in der vorliegenden Studie dieses von uns etablierte präklinische Modell verwendet, um anti-mikrobielle und anti-inflammatorische Wirkungen von Carvacrol als Option für die Prophylaxe und Therapie der Campylobacteriose zu untersuchen.

Carvacrol wurde vier Tage vor der Infektion als eine prophylaktische Behandlung über das Trinkwasser in einer Konzentration von 500 mg/L angewendet. Bis zum Tag 6 nach der Infektion konnte die Carvacrol-Vorbehandlung die intestinalen *C. jejuni*-Lasten um bis zu 2 Log-Stufen senken. Viele *in vitro*-Studien berichteten über antimikrobielle Wirkungen von Carvacrol gegenüber Pathogenen, die vor allem durch Lebensmittel übertragen werden können, wie zum Beispiel *C. jejuni* [52,81,82], *Salmonella* spp. [82,83], aber auch *Escherichia coli* [84,85]. Eine weitere Studie konnte zeigen, dass Carvacrol in der Lage ist, die Kolonisierung und Biofilmbildung von *Campylobacter* in Hühnern zu reduzieren [53,81].

Unsere Untersuchungen haben gezeigt, dass die Carvacrol-Behandlung die klinische Symptomatik der Campylobacteriose (inklusive blutigem Durchfall) lindern kann. Der Grund dafür könnte die Wirkung von Carvacrol auf die Virulenz von *C. jejuni* durch Hemmung der pathogenen Motilität, Zellanhaftung, Gewebsinvasion und Toxin-Produktion sein, die u.a. durch Hemmung der bakteriellen ATP-Produktion bewirkt wird, die zum bakteriellen Zelltod führt [52].

Die weniger stark ausgeprägte *C. jejuni*-induzierte Apoptose und verstärkte Proliferation der Darmzellen kennzeichneten eine gelinderte Campylobacteriose bei den Carvacrol-behandelten Mäusen. Die durch *C. jejuni* verursachten entzündungsfördernden Immunantworten des Darms wurde ebenfalls durch die Carvacrol-Behandlung gehemmt. Dies spiegelte sich in geringeren T- und B-Lymphozyten-Zahlen in der Schleimhaut und der Lamina propria von Dick- und des Dünndarms sowie in niedrigeren Konzentrationen von entzündungsfördernden Mediatoren einschließlich TNF und IL-6 wider.

Die Ergebnisse von weiteren *in vitro*-Studien zeigten ebenso, dass eine Carvacrol-Behandlung bei dendritischen Zellen und Makrophagen zu einer weniger ausgeprägten TNF und IFN-γ-Sekretion führte [86]. Des Weiteren reduzierte Carvacrol die LPS-induzierte Expression der pro-inflammatorischen Zytokine TNF und IL-6 *in vivo* [87,88].

In unserer aktuellen Studie konnten wir zudem zeigen, dass die entzündungshemmenden Eigenschaften von exogenem Carvacrol nicht auf den Intestinaltrakt beschränkt war. So konnten etwa weniger stark ausgeprägte apoptoische Zellreaktionen und niedrigere TNF- und IFN-γ-Konzentrationen in extraintestinalen Organen wie Leber, Nieren und Lunge bei

Carvacrol- im Vergleich zu PLC behandelten Mäusen beobachtet werden. Eine frühere Studie konnte bereits zeigen, dass die Applikation von Carvacrol während einer LPS-induzierter Endotoxinämie und akuter Lungenverletzung zu einer weniger stark ausgeprägten Sekretion von pro-inflammatorischen Zytokinen einschließlich TNF und IL-6 führte und dadurch die Überlebensrate der Mäuse stieg [89]. Interessanterweise konnte in unserer Arbeit die entzündungshemmende Wirkung von Carvacrol während einer akuten Campylobacteriose auch systemisch beobachtet werden, zumal die Serumkonzentrationen von TNF, IFN-γ, MCP-1 und IL-6 in Carvacrol im Vergleich zu mit PLC behandelten *C. jejuni*-infizierten Mäusen niedriger waren.

Zusammenfassend wirkt Carvacrol durch die Senkung der intestinalen Pathogen-Lasten und somit einer geringerer LOS-Exposition, wodurch die pro-inflammatorische Mediatorsekretion deutlich reduziert wurde und es den *C. jejuni* infizierten Mäusen klinisch wesentlich besser ging. Zusätzlich veranlassen die entzündungshemmenden Effekte von Carvacrol und die Beeinträchtigung verschiedener *C. jejuni* Virulenz-Faktoren eine geringere Rekrutierung von Immunzellen, eine geringere Sekretion der pro-inflammatorischen Mediatoren, weniger stark ausgeprägte Zellapoptose, aber verstärkte gegenregulatorische Zellproliferation /-regeneration, verminderte Translokation des Pathogens und geringere extraintestinale einschließlich systemische Immunantworten, die in der Konsequenz zu dem besseren klinischen Gesamtzustand beitragen.

4.3 Auswirkung der Vitamin D-Vorbehandlung in *C. jejuni*-infizierten, sekundär abiotischen IL-10$^{-/-}$ Mäusen

Aufgrund der positiven Wirkungen von Vitamin D auf das Immunsystem wird dessen Einnahme als Nahrungsergänzungsmittel derzeit als vielversprechende Option für die Zusatzbehandlung und/oder Prophylaxe verschiedener immunpathologischer Morbiditäten einschließlich bestimmter Infektionskrankheiten, Darmentzündungen [90] und sogar bei SARS-CoV-2 [91] empfohlen.

In der vorliegenden Interventionsstudie blieb die intestinale Pathogenbelastung (10^9 *C. jejuni* pro Gramm Fäzes) in infizierten Mäusen durch die prophylaktische Applikation von synthetischem Vitamin D unbeeinflusst. Vorherige Studien zeigten, dass die Wirkungen von Vitamin D während einer gastrointestinalen Infektion mit verschiedenen Bakterienarten wie etwa *Salmonella* [92,93] eher auf die immunmodulatorischen als auf die direkten antimikrobiellen Eigenschaften des Steroidhormons zurückzuführen sind.

Durch die hohen intestinalen Pathogenlasten zeigten die mit Vitamin D behandelten Mäuse im Vergleich zu PLC-Kontrollen am Ende des Beobachtungszeitraums eine vergleichbare makroskopische Erkrankung, dennoch litten sie während der Entwicklung der Campylobacteriose weniger unter Durchfall. Das makroskopische Ergebnis, insbesondere bei

einem derart per-akuten, nicht selbstlimitierenden Darminfektions- und -entzündungsmodell, beruht auf dem Gesamteffekt vieler verschiedener intestinaler, extraintestinaler und systemischer Ereignisse innerhalb dieses Inflammationssyndroms [94].

Des Weiteren hemmte Vitamin D die *C. jejuni*-induzierte Apoptose von Kolonepithelzellen, während deren Proliferation und Regeneration im Gegenzug eher stärker ausfiel. Diese Beobachtung war im Einklang mit der entzündungsregulierenden Wirkung von Vitamin D in dem Darmepithel [95] und in Nieren [96], in deren Zuge die Apoptose unterdrückt wird. Die weniger ausgeprägte Apoptose nach Vitamin D-Behandlung ging einher mit verminderten *C. jejuni*-induzierten Immunzellreaktionen, die durch mehrere Studien bestätigt werden, die zeigen, dass Vitamin D sowohl die angeborene als auch die adaptive Immunsystem reguliert [97,98]. In unserer Studie konnte eine geringere Anzahl von angeborenen Immunzellpopulationen wie Makrophagen und Monozyten in der Darmschleimhaut und der Lamina propria von *C. jejuni*-infizierten Mäusen beobachtet werden. Es ist bekannt, dass die durch Vitamin D verursachte Stimulation von Makrophagen und dendritischen Zellen zu einer verminderten pro-inflammatorischen Mediatorsekretion führt [99].

Die Anzahl der T-Lymphozyten in der Darmschleimhaut war in Vitamin D-behandelten Mäusen mit *C. jejuni*-induzierter Enterokolitis im Vergleich zu PLC-behandelten Mäusen niedriger. Tatsächlich wurde in vorherigen Studien gezeigt, dass Vitamin D direkte (weniger ausgeprägte T-Zell-Proliferation) und indirekte (reduzierte Sekretion von pro-inflammatorischen Zytokinen) Wirkungen auf T-Zellen hat, die anschließend zu einer Verbesserung der Entzündung führen [100-102]. Bemerkenswerterweise war die mit Vitamin D assoziierte verminderte pro-inflammatorische Mediatorsekretion nicht auf den Dickdarm beschränkt. Tatsächlich induzierte *C. jejuni* eine erhöhte Sekretion von IL-6, MCP-1 und IFN-γ im terminalen Ileum von Mäusen aus der PLC-Kontroll-, jedoch nicht aus der Vitamin D-Behandlungsgruppe.

Es ist bekannt, dass die Infektion mit *C. jejuni* zu einer Beeinträchtigung der epithelialen Barriere-Funktion führt, die die pathogene Translokation vom entzündeten Darm in extraintestinale einschließlich systemischer Kompartimente erleichtert [26,73]. Angesichts der Tatsache, dass Vitamin D die Integrität der epithelialen Barrierefunktion fördert [103], haben wir in unserer präklinischen Studie mögliche Vitamin D-vermittelte Effekte auf pathogene Translokationsfrequenzen untersucht. Unsere Ergebnisse zeigen, dass *C. jejuni* nach der Vitamin D-Behandlung mit einer geringeren Häufigkeit aus den Nieren und der Milz infizierter Mäuse kultiviert werden konnte, während die Pathogen-Isolierungsraten in MLN, Leber und Lunge in etwas mit der PLC-Gruppe vergleichbar war. Bemerkenswerterweise blieben alle Blutkulturen unabhängig vom Behandlungsschema *C. jejuni*-kultur-negativ.

Nichtdestotrotz gingen die beobachteten entzündungshemmenden Wirkungen am ehestens auf die Anwendung von Vitamin D zurück, die zu einer weniger beeinträchtigten epithelialen Barriere-Funktion des Kolons führte. Dies wiederum verringerte sehr wahrscheinlich das

Risiko der Ausbreitung sowohl lebensfähiger Bakterien als auch löslicher Bakterienmoleküle wie etwa *C. jejuni*-LOS. Es wird daher angenommen, dass die mit Vitamin D verbundenen entzündungshemmenden Wirkungen insbesondere weitere bakterieninduzierte Schäden in diesem akuten *C. jejuni*-induzierten Entzündungsmodell verhindern.
Somit liefert diese präklinische Interventionsstudie Hinweise darauf, dass die prophylaktische perorale Anwendung von Vitamin D im hier angewendeten klinischen Mausmodell die intestinalen und extraintestinalen Entzündungsreaktionen während der akuten Campylobacteriose dämpft. Weitere Studien sind erforderlich, um geeignete Vitamin D-Dosen zur Vorbeugung und Bekämpfung bestimmter gastrointestinaler Infektionskrankheiten beim Menschen zu definieren.

4.4 Fazit und Ausblick

Die oben beschriebenen präklinischen Interventionsstudien im murinen *Camplyobacter jejuni*-Infektions- und Inflammationsmodell bestätigen die Annahme, dass eine prophylaktische Behandlung mit Vitamin C, Carvacrol oder Vitamin D das Krankheitsbild der Campylobacteriose auf verschiedenen Ebenen verbessern kann. Die Ergebnisse belegen die immun-modulierenden Eigenschaften der oral verabreichten Substanzen während einer akuten *C. jejuni*-induzierten Enterokolitis. Während die Behandlung mit Vitamin C oder Carvacrol die Pathogenlasten reduzierte und dadurch sowohl antimikrobiell als auch immunregulierend wirkte, zeigte die Behandlung mit Vitamin D eine eher anti-inflammatorische Wirkung im Rahmen der *C. jejuni*-Infektion. Um das Syndrom der Campylobacteriose möglichst auf verschiedenen Ebenen positiv zu beeinflussen, wäre etwa eine Kombination von Vitamin C, D und Carvacrol als vielsprechende Behandlungsoption denkbar.
Trotz einer Linderung der *C. jejuni*-induzierten Symptomen nach einer Behandlung mit einer einzelnen Substanz wurden extraintestinale Kollateralschäden nach einer *C. jejuni*-Infektion beobachtet. Das Zirkulieren von löslichen Bakterienmolekülen wie etwa *C. jejuni*-LOS, vom entzündeten Darm ausgehend über den Kreislauf, könnte eine mögliche Erklärung für diese Beobachtung sein. Das LOS bzw. LPS der Gram-negativen Bakterien bindet sich an TLR-4 und löst somit eine Signalkaskade aus, die zu einer Inflammationsreaktions führt [5]. Allerdings ist bekannt, dass es zwischen der Aktivierung von murinem und menschlichem TLR-4 durchaus Unterschiede gibt, die höchstwahrscheinlich auf das LPS/LOS-Erkennungsmuster des Rezeptorkomplexes zurückzuführen sind [104].
Dennoch ist einer der Hauptunterschiede zwischen murinem und humanem Organismus die Kolonisationsresistenz der Wildtyp-Maus gegenüber *C. jejuni*. Daher wurde in unseren Studien das von der Arbeitsgruppe etablierte Mausmodell, die sekundär abiotische IL-$10^{-/-}$ Maus [26], verwendet, welches durch die Antibiotika-Vorbehandlung keine murine Flora beherbergt. Dieser Aspekt und das Fehlen des IL-10-Gens ermöglichen die Kolonisation und

darauffolgende Infektion von *C. jejuni* in murinen Gastrointestinaltrakt. Wie schon bereits von der Arbeitsgruppe beschrieben, können diese sekundär abiotischen Mäuse ebenfalls mit einer humanen Darmmikrobiota erfolgreich kolonisiert werden [105,106]. Somit wird eine human-ähnliche („humanisierte“) Situation im Rahmen der Pathogen-Wirts-Interaktionen dargestellt. Hierbei könnten die Resultate einer prophylaktischen Behandlung der (bezüglich ihrer Darmmikrobiota) „humanisierten“ Mäuse mit den Substanzen, gefolgt von der *C. jejuni*-Infektion, besser auf die humane Campylobacteriose übertragen werden.

Zusammenfassend, liefern diese präklinische Interventionsstudien Hinweise darauf, dass die prophylaktische perorale Anwendung von Vitamin C, Carvacrol und Vitamin D im hier angewendeten klinischen Mausmodell die intestinalen und extraintestinalen Entzündungsreaktionen während der akuten Campylobacteriose reduzieren kann. Weitere Studien sind erforderlich, um i) die direkte Wirkung der Substanzen gegen *C. jejuni* auf molekulare Ebene zu untersuchen und ii) die geeigneten Dosen zur Vorbeugung und Bekämpfung bestimmter gastrointestinaler Infektionskrankheiten beim Menschen zu definieren.

5. Literaturverzeichnis

1. EFSA. European Food Safety Authority. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013. EFSA J 2015;13. . **2013**, doi:10.2903/j., doi:doi:10.2903/j.
2. EFSA. European Food Safety Authority. European Centre for Disease, Prevention Control,The European Union One Health 2018 Zoonoses Report. *EFSA Journal* **2019**, *17*, e05926, doi:10.2903/j.efsa.2019.5926.
3. Fischer, G.H.; Paterek, E. *Campylobacter*. In *StatPearls*, StatPearls Publishing Copyright © 2020, StatPearls Publishing LLC.: Treasure Island (FL), 2020.
4. Backert, S.; Tegtmeyer, N.; Cróinín, T.Ó.; Boehm, M.; Heimesaat, M.M. Human campylobacteriosis. In *Campylobacter*, Klein, G., Ed. Academic Press: 2017; 10.1016/b978-0-12-803623-5.00001-0pp. 1-25.
5. Mousavi, S.; Bereswill, S.; Heimesaat, M.M. Novel Clinical *Campylobacter jejuni* Infection Models Based on Sensitization of Mice to Lipooligosaccharide, a Major Bacterial Factor Triggering Innate Immune Responses in Human Campylobacteriosis. *Microorganisms* **2020**, *8*, doi:10.3390/microorganisms8040482.
6. Korolik, V. The role of chemotaxis during *Campylobacter jejuni* colonisation and pathogenesis. *Curr Opin Microbiol* **2019**, *47*, 32-37, doi:10.1016/j.mib.2018.11.001.
7. Ellström, P.; Hansson, I.; Nilsson, A.; Rautelin, H.; Olsson Engvall, E. Lipooligosaccharide locus classes and putative virulence genes among chicken and human Campylobacter jejuni isolates. *BMC Microbiology* **2016**, *16*, 116, doi:10.1186/s12866-016-0740-5.
8. Gardner, T.J.; Fitzgerald, C.; Xavier, C.; Klein, R.; Pruckler, J.; Stroika, S.; McLaughlin, J.B. Outbreak of campylobacteriosis associated with consumption of raw peas. *Clin Infect Dis* **2011**, *53*, 26-32, doi:10.1093/cid/cir249.
9. Brandl, M.T.; Haxo, A.F.; Bates, A.H.; Mandrell, R.E. Comparison of survival of *Campylobacter jejuni* in the phyllosphere with that in the rhizosphere of spinach and radish plants. *Appl Environ Microbiol* **2004**, *70*, 1182-1189, doi:10.1128/aem.70.2.1182-1189.2004.
10. Facciolà, A.; Riso, R.; Avventuroso, E.; Visalli, G.; Delia, S.A.; Laganà, P. *Campylobacter:* from microbiology to prevention. *J Prev Med Hyg* **2017**, *58*, E79-e92.
11. S2k-Leitlinie. Akute infektiöse Gastroenteritis im Säuglings-, Kindes- und Jugendalter; AWMF Registernummer 068/003; Version 04.05.2019. **2019**.
12. Maldonado, R.F.; Sá-Correia, I.; Valvano, M.A. Lipopolysaccharide modification in Gram-negative bacteria during chronic infection. *FEMS microbiology reviews* **2016**, *40*, 480-493, doi:10.1093/femsre/fuw007.
13. Mortensen, N.P.; Kuijf, M.L.; Ang, C.W.; Schiellerup, P.; Krogfelt, K.A.; Jacobs, B.C.; van Belkum, A.; Endtz, H.P.; Bergman, M.P. Sialylation of *Campylobacter jejuni* lipo-oligosaccharides is associated with severe gastro-enteritis and reactive arthritis. *Microbes Infect* **2009**, *11*, 988-994, doi:10.1016/j.micinf.2009.07.004.
14. Hameed, A.; Woodacre, A.; Machado, L.R.; Marsden, G.L. An Updated Classification System and Review of the Lipooligosaccharide Biosynthesis Gene Locus in *Campylobacter jejuni*. *Front Microbiol* **2020**, *11*, 677, doi:10.3389/fmicb.2020.00677.
15. Houliston, R.S.; Vinogradov, E.; Dzieciatkowska, M.; Li, J.; St Michael, F.; Karwaski, M.F.; Brochu, D.; Jarrell, H.C.; Parker, C.T.; Yuki, N., et al. Lipooligosaccharide of *Campylobacter jejuni*: similarity with multiple types of mammalian glycans beyond gangliosides. *J Biol Chem* **2011**, *286*, 12361-12370, doi:10.1074/jbc.M110.181750.
16. Gilbert, M.; Godschalk, P.C.; Karwaski, M.F.; Ang, C.W.; van Belkum, A.; Li, J.; Wakarchuk, W.W.; Endtz, H.P. Evidence for acquisition of the lipooligosaccharide biosynthesis locus in *Campylobacter jejuni* GB11, a strain isolated from a patient with Guillain-Barré syndrome, by horizontal exchange. *Infect Immun* **2004**, *72*, 1162-1165, doi:10.1128/iai.72.2.1162-1165.2004.
17. Habib, I.; Louwen, R.; Uyttendaele, M.; Houf, K.; Vandenberg, O.; Nieuwenhuis, E.E.; Miller, W.G.; van Belkum, A.; De Zutter, L. Correlation between genotypic diversity, lipooligosaccharide gene locus class variation, and caco-2 cell invasion potential of

*Campylobacter jejuni i*solates from chicken meat and humans: contribution to virulotyping. *Appl Environ Microbiol* **2009**, *75*, 4277-4288, doi:10.1128/aem.02269-08.
18. Riddle, M.S.; Guerry, P. Status of vaccine research and development for Campylobacter jejuni. *Vaccine* **2016**, *34*, 2903-2906, doi:10.1016/j.vaccine.2016.02.080.
19. Heimesaat, M.M.; Bereswill, S. Murine infection models for the investigation of *Campylobacter jejuni*--host interactions and pathogenicity. *Berliner und Munchener Tierarztliche Wochenschrift* **2015**, *128*, 98-103.
20. van Spreeuwel, J.P.; Duursma, G.C.; Meijer, C.J.; Bax, R.; Rosekrans, P.C.; Lindeman, J. *Campylobacter* colitis: histological immunohistochemical and ultrastructural findings. *Gut* **1985**, *26*, 945-951, doi:10.1136/gut.26.9.945.
21. Flamm, H. [The Austrian pediatrician Theodor Escherich as bacteriologist and social hygienist: The 100th anniversary of his death on February 15th, 1911]. *Wiener klinische Wochenschrift* **2011**, *123*, 157-171, doi:10.1007/s00508-011-1533-3.
22. Stahl, M.; Graef, F.A.; Vallance, B.A. Mouse Models for Campylobacter jejuni Colonization and Infection. *Methods Mol Biol* **2017**, *1512*, 171-188, doi:10.1007/978-1-4939-6536-6_15.
23. Mansfield, L.S.; Bell, J.A.; Wilson, D.L.; Murphy, A.J.; Elsheikha, H.M.; Rathinam, V.A.; Fierro, B.R.; Linz, J.E.; Young, V.B. C57BL/6 and congenic interleukin-10-deficient mice can serve as models of *Campylobacter jejuni* colonization and enteritis. *Infect Immun* **2007**, *75*, 1099-1115, doi:10.1128/IAI.00833-06.
24. Lippert, E.; Karrasch, T.; Sun, X.; Allard, B.; Herfarth, H.H.; Threadgill, D.; Jobin, C. Gnotobiotic IL-10; NF-kappaB mice develop rapid and severe colitis following *Campylobacter jejuni* infection. *PloS one* **2009**, *4*, e7413, doi:10.1371/journal.pone.0007413.
25. Kühn, R.; Löhler, J.; Rennick, D.; Rajewsky, K.; Müller, W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* **1993**, *75*, 263-274, doi:10.1016/0092-8674(93)80068-p.
26. Haag, L.M.; Fischer, A.; Otto, B.; Plickert, R.; Kuhl, A.A.; Gobel, U.B.; Bereswill, S.; Heimesaat, M.M. *Campylobacter jejuni* induces acute enterocolitis in gnotobiotic IL-10-/- mice via Toll-like-receptor-2 and -4 signaling. *PloS one* **2012**, *7*, e40761, doi:10.1371/journal.pone.0040761.
27. Klancnik, A.; Mozina, S.S.; Zhang, Q. Anti-Campylobacter activities and resistance mechanisms of natural phenolic compounds in Campylobacter. *PLoS One* **2012**, *7*, e51800, doi:10.1371/journal.pone.0051800.
28. Heimesaat, M.M.; Mousavi, S.; Escher, U.; Lobo de Sá, F.D.; Peh, E.; Schulzke, J.-D.; Kittler, S.; Bücker, R.; Bereswill, S. Resveratrol Alleviates Acute *Campylobacter jejuni* Induced Enterocolitis in a Preclinical Murine Intervention Study. *Microorganisms* **2020**, *8*, 1858.
29. Lobo de Sá, F.D.; Butkevych, E.; Nattramilarasu, P.K.; Fromm, A.; Mousavi, S.; Moos, V.; Golz, J.C.; Stingl, K.; Kittler, S.; Seinige, D. Curcumin mitigates immune-induced epithelial barrier dysfunction by *Campylobacter jejuni*. *International journal of molecular sciences* **2019**, *20*, 4830.
30. Heimesaat, M.M.; Mousavi, S.; Weschka, D.; Bereswill, S. Anti-Pathogenic and Immune-Modulatory Effects of Peroral Treatment with Cardamom Essential Oil in Acute Murine Campylobacteriosis. *Microorganisms* **2021**, *9*, 169, doi:10.3390/microorganisms9010169.
31. Mousavi, S.; Weschka, D.; Bereswill, S.; Heimesaat, M.M. Preclinical Evaluation of Oral Urolithin-A for the Treatment of Acute Campylobacteriosis in *Campylobacter jejuni* Infected Microbiota-Depleted IL-10-/-Mice. *Pathogens* **2020**, *10*, 7.
32. Mousavi, S.; Bereswill, S.; Heimesaat, M.M. Immunomodulatory and Antimicrobial Effects of Vitamin C. *European Journal of Microbiology and Immunolog* **2019**, 10.1556/1886.2019.00016, doi:10.1556/1886.2019.00016.

33. Mousavi, S.; Escher, U.; Thunhorst, E.; Kittler, S.; Kehrenberg, C.; Bereswill, S.; Heimesaat, M.M. Vitamin C alleviates acute enterocolitis in *Campylobacter jejuni* infected mice. *Sci Rep* **2020**, *10*, 2921, doi:10.1038/s41598-020-59890-8.
34. Smirnoff, N. L-ascorbic acid biosynthesis. *Vitamins and hormones* **2001**, *61*, 241-266, doi:10.1016/s0083-6729(01)61008-2.
35. Linster, C.L.; Van Schaftingen, E. Vitamin C. Biosynthesis, recycling and degradation in mammals. *FEBS J* **2007**, *274*, 1-22, doi:10.1111/j.1742-4658.2006.05607.x.
36. Lykkesfeldt, J.; Michels, A.J.; Frei, B. Vitamin C. *Adv Nutr* **2014**, *5*, 16-18, doi:10.3945/an.113.005157.
37. Sorice, A.; Guerriero, E.; Capone, F.; Colonna, G.; Castello, G.; Costantini, S. Ascorbic acid: its role in immune system and chronic inflammation diseases. *Mini Rev Med Chem* **2014**, *14*, 444-452.
38. Syal, K.; Chatterji, D. Vitamin C: A Natural Inhibitor of Cell Wall Functions and Stress Response in Mycobacteria. *Adv Exp Med Biol* **2018**, *1112*, 321-332, doi:10.1007/978-981-13-3065-0_22.
39. van Asbeck, B.S.; Marcelis, J.H.; Marx, J.J.; Struyvenberg, A.; van Kats, J.H.; Verhoef, J. Inhibition of bacterial multiplication by the iron chelator deferoxamine: potentiating effect of ascorbic acid. *Eur J Clin Microbiol* **1983**, *2*, 426-431.
40. Tajkarimi, M.; Ibrahim, S.A. Antimicrobial activity of ascorbic acid alone or in combination with lactic acid on Escherichia coli O157: H7 in laboratory medium and carrot juice. *Food Control* **2011**, *22*, 801-804.
41. Sezikli, M.; Cetinkaya, Z.A.; Guzelbulut, F.; Cimen, B.; Ozcan, O.; Ozkara, S.; Yesil, A.; Gumrukcu, G.; Ipcioglu, O.M.; Sezikli, H., et al. Effects of alpha tocopherol and ascorbic acid on Helicobacter pylori colonization and the severity of gastric inflammation. *Helicobacter* **2012**, *17*, 127-132, doi:10.1111/j.1523-5378.2011.00925.x.
42. Sezikli, M.; Çetinkaya, Z.A.; Sezikli, H.; Güzelbulut, F.; Tiftikçi, A.; Tüzün İnce, A.; Gökden, Y.; Yaşar, B.; Atalay, S.; Övünç Kurdaş, O. Oxidative stress in Helicobacter pylori infection: does supplementation with vitamins C and E increase the eradication rate? *Helicobacter* **2009**, *14*, 280-285.
43. Hernandez-Patlan, D.; Solis-Cruz, B.; Mendez-Albores, A.; Latorre, J.D.; Hernandez-Velasco, X.; Tellez, G.; Lopez-Arellano, R. Comparison of PrestoBlue((R)) and plating method to evaluate antimicrobial activity of ascorbic acid, boric acid and curcumin in an in vitro gastrointestinal model. *J Appl Microbiol* **2018**, *124*, 423-430, doi:10.1111/jam.13659.
44. Hernandez-Patlan, D.; Solis-Cruz, B.; Patrin Pontin, K.; Latorre, J.D.; Baxter, M.F.A.; Hernandez-Velasco, X.; Merino-Guzman, R.; Mendez-Albores, A.; Hargis, B.M.; Lopez-Arellano, R., et al. Evaluation of the Dietary Supplementation of a Formulation Containing Ascorbic Acid and a Solid Dispersion of Curcumin with Boric Acid against Salmonella Enteritidis and Necrotic Enteritis in Broiler Chickens. *Animals (Basel)* **2019**, *9*, doi:10.3390/ani9040184.
45. Vilchèze, C.; Hartman, T.; Weinrick, B.; Jacobs, W.R. *Mycobacterium tuberculosis* is extraordinarily sensitive to killing by a vitamin C-induced Fenton reaction. *Nature Communications* **2013**, *4*, 1881, doi:10.1038/ncomms2898.
46. Fletcher, R.D.; Albers, A.C.; Chen, A.K.; Albertson, J.N., Jr. Ascorbic acid inhibition of Campylobacter jejuni growth. *Applied and environmental microbiology* **1983**, *45*, 792-795.
47. Juven, B.J.; Kanner, J. Effect of ascorbic, isoascorbic and dehydroascorbic acids on the growth and survival of Campylobacter jejuni. *J Appl Bacteriol* **1986**, *61*, 339-345.
48. Juven, B.J.; Kanner, J.; Weisslowicz, H.; Harel, S. Effect of Ascorbic and Isoascorbic acids on Survival of Campylobacter jejuni in Poultry Meat (1). *J Food Prot* **1988**, *51*, 436-437, doi:10.4315/0362-028X-51.6.436.
49. Sharifi-Rad, M.; Varoni, E.M.; Iriti, M.; Martorell, M.; Setzer, W.N.; Del Mar Contreras, M.; Salehi, B.; Soltani-Nejad, A.; Rajabi, S.; Tajbakhsh, M., et al. Carvacrol and human health: A comprehensive review. *Phytother Res* **2018**, *32*, 1675-1687, doi:10.1002/ptr.6103.

50. Mousavi, S.; Schmidt, A.M.; Escher, U.; Kittler, S.; Kehrenberg, C.; Thunhorst, E.; Bereswill, S.; Heimesaat, M.M. Carvacrol ameliorates acute campylobacteriosis in a clinical murine infection model. *Gut Pathog* **2020**, *12*, 2, doi:10.1186/s13099-019-0343-4.
51. Ayres Cacciatore, F.; Dalmás, M.; Maders, C.; Ataíde Isaía, H.; Brandelli, A.; da Silva Malheiros, P. Carvacrol encapsulation into nanostructures: Characterization and antimicrobial activity against foodborne pathogens adhered to stainless steel. *Food Res Int* **2020**, *133*, 109143, doi:10.1016/j.foodres.2020.109143.
52. Upadhyay, A.; Arsi, K.; Wagle, B.R.; Upadhyaya, I.; Shrestha, S.; Donoghue, A.M.; Donoghue, D.J. Trans-Cinnamaldehyde, Carvacrol, and Eugenol Reduce *Campylobacter jejuni* Colonization Factors and Expression of Virulence Genes in Vitro. *Frontiers in Microbiology* **2017**, *8*, doi:10.3389/fmicb.2017.00713.
53. Wagle, B.R.; Donoghue, A.M.; Shrestha, S.; Upadhyaya, I.; Arsi, K.; Gupta, A.; Liyanage, R.; Rath, N.C.; Donoghue, D.J.; Upadhyay, A. Carvacrol attenuates *Campylobacter jejuni* colonization factors and proteome critical for persistence in the chicken gut. *Poult Sci* **2020**, *99*, 4566-4577, doi:10.1016/j.psj.2020.06.020.
54. Di Pasqua, R.; Hoskins, N.; Betts, G.; Mauriello, G. Changes in membrane fatty acids composition of microbial cells induced by addiction of thymol, carvacrol, limonene, cinnamaldehyde, and eugenol in the growing media. *J Agric Food Chem* **2006**, *54*, 2745-2749, doi:10.1021/jf052722l.
55. van Alphen, L.B.; Burt, S.A.; Veenendaal, A.K.; Bleumink-Pluym, N.M.; van Putten, J.P. The natural antimicrobial carvacrol inhibits *Campylobacter jejuni* motility and infection of epithelial cells. *PLoS One* **2012**, *7*, e45343, doi:10.1371/journal.pone.0045343.
56. Gill, A.O.; Holley, R.A. Disruption of *Escherichia coli, Listeria monocytogenes* and *Lactobacillus sakei* cellular membranes by plant oil aromatics. *Int J Food Microbiol* **2006**, *108*, 1-9, doi:10.1016/j.ijfoodmicro.2005.10.009.
57. Allaoua, M.; Etienne, P.; Noirot, V.; Carayon, J.L.; Tene, N.; Bonnafe, E.; Treilhou, M. Pharmacokinetic and antimicrobial activity of a new carvacrol-based product against a human pathogen, Campylobacter jejuni. *J Appl Microbiol* **2018**, *125*, 1162-1174, doi:10.1111/jam.13915.
58. Shrestha, S.; Wagle, B.R.; Upadhyay, A.; Arsi, K.; Donoghue, D.J.; Donoghue, A.M. Carvacrol antimicrobial wash treatments reduce Campylobacter jejuni and aerobic bacteria on broiler chicken skin. *Poult Sci* **2019**, *98*, 4073-4083, doi:10.3382/ps/pez198.
59. Szott, V.; Reichelt, B.; Alter, T.; Friese, A.; Roesler, U. In vivo efficacy of carvacrol on Campylobacter jejuni prevalence in broiler chickens during an entire fattening period. *Eur J Microbiol Immunol (Bp)* **2020**, *10*, 131-138, doi:10.1556/1886.2020.00011.
60. Provvedini, D.M.; Tsoukas, C.D.; Deftos, L.J.; Manolagas, S.C. 1,25-dihydroxyvitamin D3 receptors in human leukocytes. *Science (New York, N.Y.)* **1983**, *221*, 1181-1183.
61. Karlic, H.; Varga, F. Impact of vitamin D metabolism on clinical epigenetics. *Clin Epigenetics* **2011**, *2*, 55-61, doi:10.1007/s13148-011-0021-y.
62. Golpour, A.; Bereswill, S.; Heimesaat, M.M. Antimicrobial and Immune-Modulatory Effects of Vitamin D Provide Promising Antibiotics-Independent Approaches to Tackle Bacterial Infections - Lessons Learnt from a Literature Survey. *Eur J Microbiol Immunol (Bp)* **2019**, *9*, 80-87, doi:10.1556/1886.2019.00014.
63. Rook, G.A.; Steele, J.; Fraher, L.; Barker, S.; Karmali, R.; O'Riordan, J.; Stanford, J. Vitamin D3, gamma interferon, and control of proliferation of *Mycobacterium tuberculosis* by human monocytes. *Immunology* **1986**, *57*, 159-163.
64. Youssef, D.A.; Miller, C.W.; El-Abbassi, A.M.; Cutchins, D.C.; Cutchins, C.; Grant, W.B.; Peiris, A.N. Antimicrobial implications of vitamin D. *Dermatoendocrinol* **2011**, *3*, 220-229, doi:10.4161/derm.3.4.15027.
65. Mousavi, S.; Lobo de Sa, F.D.; Schulzke, J.D.; Bucker, R.; Bereswill, S.; Heimesaat, M.M. Vitamin D in Acute Campylobacteriosis-Results From an Intervention Study Applying a Clinical Campylobacter jejuni Induced Enterocolitis Model. *Frontiers in immunology* **2019**, *10*, 2094, doi:10.3389/fimmu.2019.02094.

66. Saputo, S.; Faustoferri, R.C.; Quivey, R.G., Jr. Vitamin D Compounds Are Bactericidal against Streptococcus mutans and Target the Bacitracin-Associated Efflux System. *Antimicrob Agents Chemother* **2018**, *62*, doi:10.1128/AAC.01675-17.
67. Kawaura, A.; Takeda, E.; Tanida, N.; Nakagawa, K.; Yamamoto, H.; Sawada, K.; Okano, T. Inhibitory effect of long term 1α-hydroxyvitamin D3 administration on Helicobacter pylori infection. *Journal of Clinical Biochemistry and Nutrition* **2006**, *38*, 103-106.
68. Gubatan, J.; Chou, N.D.; Nielsen, O.H.; Moss, A.C. Systematic review with meta-analysis: association of vitamin D status with clinical outcomes in adult patients with inflammatory bowel disease. *Alimentary Pharmacology & Therapeutics* **2019**, *50*, 1146-1158, doi:https://doi.org/10.1111/apt.15506.
69. Garcia, P.M.; Moore, J.; Kahan, D.; Hong, M.Y. Effects of Vitamin D Supplementation on Inflammation, Colonic Cell Kinetics, and Microbiota in Colitis: A Review. *Molecules (Basel, Switzerland)* **2020**, *25*, doi:10.3390/molecules25102300.
70. Meeker, S.; Seamons, A.; Paik, J.; Treuting, P.M.; Brabb, T.; Grady, W.M.; Maggio-Price, L. Increased dietary vitamin D suppresses MAPK signaling, colitis, and colon cancer. *Cancer research* **2014**, *74*, 4398-4408, doi:10.1158/0008-5472.Can-13-2820.
71. Liu, W.; Chen, Y.; Golan, M.A.; Annunziata, M.L.; Du, J.; Dougherty, U.; Kong, J.; Musch, M.; Huang, Y.; Pekow, J., et al. Intestinal epithelial vitamin D receptor signaling inhibits experimental colitis. *The Journal of clinical investigation* **2013**, *123*, 3983-3996, doi:10.1172/JCI65842.
72. Daniel, C.; Sartory, N.A.; Zahn, N.; Radeke, H.H.; Stein, J.M. Immune modulatory treatment of trinitrobenzene sulfonic acid colitis with calcitriol is associated with a change of a T helper (Th) 1/Th17 to a Th2 and regulatory T cell profile. *J Pharmacol Exp Ther* **2008**, *324*, 23-33, doi:10.1124/jpet.107.127209.
73. Bucker, R.; Krug, S.M.; Moos, V.; Bojarski, C.; Schweiger, M.R.; Kerick, M.; Fromm, A.; Janssen, S.; Fromm, M.; Hering, N.A., et al. Campylobacter jejuni impairs sodium transport and epithelial barrier function via cytokine release in human colon. *Mucosal immunology* **2018**, *11*, 474-485, doi:10.1038/mi.2017.66.
74. v. Gagyi, J. Ueber die bactericide und antitoxische Wirkung des Vitamin C. *Journal of Molecular Medicine* **1936**, *15*, 190-195.
75. Chen, X.; Liu, R.; Liu, X.; Xu, C.; Wang, X. L-ascorbic Acid-2-Glucoside inhibits Helicobacter pylori-induced apoptosis through mitochondrial pathway in Gastric Epithelial cells. *Biomed Pharmacother* **2018**, *97*, 75-81, doi:10.1016/j.biopha.2017.10.030.
76. Sharma, P.; Raghavan, S.A.; Saini, R.; Dikshit, M. Ascorbate-mediated enhancement of reactive oxygen species generation from polymorphonuclear leukocytes: modulatory effect of nitric oxide. *J Leukoc Biol* **2004**, *75*, 1070-1078, doi:10.1189/jlb.0903415.
77. Perez-Cruz, I.; Carcamo, J.M.; Golde, D.W. Vitamin C inhibits FAS-induced apoptosis in monocytes and U937 cells. *Blood* **2003**, *102*, 336-343, doi:10.1182/blood-2002-11-3559.
78. Hartel, C.; Strunk, T.; Bucsky, P.; Schultz, C. Effects of vitamin C on intracytoplasmic cytokine production in human whole blood monocytes and lymphocytes. *Cytokine* **2004**, *27*, 101-106, doi:10.1016/j.cyto.2004.02.004.
79. Maeng, H.G.; Lim, H.; Jeong, Y.J.; Woo, A.; Kang, J.S.; Lee, W.J.; Hwang, Y.I. Vitamin C enters mouse T cells as dehydroascorbic acid in vitro and does not recapitulate in vivo vitamin C effects. *Immunobiology* **2009**, *214*, 311-320, doi:10.1016/j.imbio.2008.09.003.
80. Dey, S.; Bishayi, B. Killing of S. aureus in murine peritoneal macrophages by Ascorbic acid along with antibiotics Chloramphenicol or Ofloxacin: Correlation with inflammation. *Microbial pathogenesis* **2018**, *115*, 239-250, doi:10.1016/j.micpath.2017.12.048.
81. Kelly, C.; Gundogdu, O.; Pircalabioru, G.; Cean, A.; Scates, P.; Linton, M.; Pinkerton, L.; Magowan, E.; Stef, L.; Simiz, E., et al. The In Vitro and In Vivo Effect of Carvacrol in Preventing Campylobacter Infection, Colonization and in Improving Productivity of

Chicken Broilers. *Foodborne Pathog Dis* **2017**, *14*, 341-349, doi:10.1089/fpd.2016.2265.
82. Ozogul, Y.; Kuley, E.; Ucar, Y.; Ozogul, F. Antimicrobial Impacts of Essential Oils on Food Borne-Pathogens. *Recent patents on food, nutrition & agriculture* **2015**, *7*, 53-61, doi:10.2174/2212798407666150615112153.
83. Beaubrun, J.J.; Addy, N.; Keltner, Z.; Farris, S.; Ewing, L.; Gopinath, G.; Hanes, D.E. Evaluation of the Impact of Varied Carvacrol Concentrations on *Salmonella* Recovery in Oregano and How Corn Oil Can Minimize the Effect of Carvacrol during Preenrichment. *J Food Prot* **2018**, *81*, 977-985, doi:10.4315/0362-028x.Jfp-17-489.
84. Khan, I.; Bahuguna, A.; Shukla, S.; Aziz, F.; Chauhan, A.K.; Ansari, M.B.; Bajpai, V.K.; Huh, Y.S.; Kang, S.C. Antimicrobial potential of the food-grade additive carvacrol against uropathogenic E*. coli* based on membrane depolarization, reactive oxygen species generation, and molecular docking analysis. *Microb Pathog* **2020**, *142*, 104046, doi:10.1016/j.micpath.2020.104046.
85. Xu, J.; Zhou, F.; Ji, B.P.; Pei, R.S.; Xu, N. The antibacterial mechanism of carvacrol and thymol against *Escherichia coli*. *Lett Appl Microbiol* **2008**, *47*, 174-179, doi:10.1111/j.1472-765X.2008.02407.x.
86. Amirghofran, Z.; Ahmadi, H.; Karimi, M.H.; Kalantar, F.; Gholijani, N.; Malek-Hosseini, Z. In vitro inhibitory effects of thymol and carvacrol on dendritic cell activation and function. *Pharm Biol* **2016**, *54*, 1125-1132, doi:10.3109/13880209.2015.1055579.
87. Somensi, N.; Rabelo, T.K.; Guimarães, A.G.; Quintans-Junior, L.J.; de Souza Araújo, A.A.; Moreira, J.C.F.; Gelain, D.P. Carvacrol suppresses LPS-induced pro-inflammatory activation in RAW 264.7 macrophages through ERK1/2 and NF-kB pathway. *Int Immunopharmacol* **2019**, *75*, 105743, doi:10.1016/j.intimp.2019.105743.
88. Liu, S.D.; Song, M.H.; Yun, W.; Lee, J.H.; Kim, H.B.; Cho, J.H. Effect of carvacrol essential oils on immune response and inflammation-related genes expression in broilers challenged by lipopolysaccharide. *Poult Sci* **2019**, *98*, 2026-2033, doi:10.3382/ps/pey575.
89. Feng, X.; Jia, A. Protective effect of carvacrol on acute lung injury induced by lipopolysaccharide in mice. *Inflammation* **2014**, *37*, 1091-1101, doi:10.1007/s10753-014-9833-1.
90. Nicholson, I.; Dalzell, A.M.; El-Matary, W. Vitamin D as a therapy for colitis: a systematic review. *J Crohns Colitis* **2012**, *6*, 405-411, doi:10.1016/j.crohns.2012.01.007.
91. Grant, W.B.; Lahore, H.; McDonnell, S.L.; Baggerly, C.A.; French, C.B.; Aliano, J.L.; Bhattoa, H.P. Evidence that Vitamin D Supplementation Could Reduce Risk of Influenza and COVID-19 Infections and Deaths. *Nutrients* **2020**, *12*, doi:10.3390/nu12040988.
92. Huang, F.C. The differential effects of 1,25-dihydroxyvitamin D3 on Salmonella-induced interleukin-8 and human beta-defensin-2 in intestinal epithelial cells. *Clin Exp Immunol* **2016**, *185*, 98-106, doi:10.1111/cei.12792.
93. Huang, F.C. Vitamin D differentially regulates Salmonella-induced intestine epithelial autophagy and interleukin-1beta expression. *World J Gastroenterol* **2016**, *22*, 10353-10363, doi:10.3748/wjg.v22.i47.10353.
94. Schmidt, A.M.; Escher, U.; Mousavi, S.; Boehm, M.; Backert, S.; Bereswill, S.; Heimesaat, M.M. Protease Activity of Campylobacter jejuni HtrA Modulates Distinct Intestinal and Systemic Immune Responses in Infected Secondary Abiotic IL-10 Deficient Mice. *Front Cell Infect Microbiol* **2019**, *9*, 79, doi:10.3389/fcimb.2019.00079.
95. He, L.; Liu, T.; Shi, Y.; Tian, F.; Hu, H.; Deb, D.K.; Chen, Y.; Bissonnette, M.; Li, Y.C. Gut Epithelial Vitamin D Receptor Regulates Microbiota-Dependent Mucosal Inflammation by Suppressing Intestinal Epithelial Cell Apoptosis. *Endocrinology* **2018**, *159*, 967-979, doi:10.1210/en.2017-00748.
96. Du, J.; Jiang, S.; Hu, Z.; Tang, S.; Sun, Y.; He, J.; Li, Z.; Yi, B.; Wang, J.; Zhang, H., et al. Vitamin D receptor activation protects against lipopolysaccharide-induced acute

kidney injury through suppression of tubular cell apoptosis. *Am J Physiol Renal Physiol* **2019**, *316*, F1068-f1077, doi:10.1152/ajprenal.00332.2018.
97. Trochoutsou, A.I.; Kloukina, V.; Samitas, K.; Xanthou, G. Vitamin-D in the Immune System: Genomic and Non-Genomic Actions. *Mini Rev Med Chem* **2015**, *15*, 953-963, doi:10.2174/1389557515666150519110830.
98. Chun, R.F.; Liu, P.T.; Modlin, R.L.; Adams, J.S.; Hewison, M. Impact of vitamin D on immune function: lessons learned from genome-wide analysis. *Front Physiol* **2014**, *5*, 151, doi:10.3389/fphys.2014.00151.
99. Bartels, L.E.; Bendix, M.; Hvas, C.L.; Jorgensen, S.P.; Agnholt, J.; Agger, R.; Dahlerup, J.F. Oral vitamin D3 supplementation reduces monocyte-derived dendritic cell maturation and cytokine production in Crohn's disease patients. *Inflammopharmacology* **2014**, *22*, 95-103, doi:10.1007/s10787-013-0197-1.
100. Cantorna, M.T.; McDaniel, K.; Bora, S.; Chen, J.; James, J. Vitamin D, immune regulation, the microbiota, and inflammatory bowel disease. *Experimental biology and medicine (Maywood, N.J.)* **2014**, *239*, 1524-1530, doi:10.1177/1535370214523890.
101. Cantorna, M.T.; Waddell, A. The vitamin D receptor turns off chronically activated T cells. *Annals of the New York Academy of Sciences* **2014**, *1317*, 70-75, doi:10.1111/nyas.12408.
102. Kongsbak, M.; Levring, T.B.; Geisler, C.; von Essen, M.R. The vitamin d receptor and T cell function. *Frontiers in immunology* **2013**, *4*, 148, doi:10.3389/fimmu.2013.00148.
103. Schwalfenberg, G.K. A review of the critical role of vitamin D in the functioning of the immune system and the clinical implications of vitamin D deficiency. *Molecular nutrition & food research* **2011**, *55*, 96-108, doi:10.1002/mnfr.201000174.
104. Vaure, C.; Liu, Y. A comparative review of toll-like receptor 4 expression and functionality in different animal species. *Frontiers in immunology* **2014**, *5*, 316, doi:10.3389/fimmu.2014.00316.
105. Bereswill, S.; Fischer, A.; Plickert, R.; Haag, L.M.; Otto, B.; Kuhl, A.A.; Dasti, J.I.; Zautner, A.E.; Munoz, M.; Loddenkemper, C., et al. Novel murine infection models provide deep insights into the "menage a trois" of *Campylobacter jejuni*, microbiota and host innate immunity. *PloS one* **2011**, *6*, e20953, doi:10.1371/journal.pone.0020953.
106. Kløve, S.; Genger, C.; Mousavi, S.; Weschka, D.; Bereswill, S.; Heimesaat, M.M. Toll-Like Receptor-4 Dependent Intestinal and Systemic Sequelae Following Peroral *Campylobacter coli* Infection of IL10 Deficient Mice Harboring a Human Gut Microbiota. *Pathogens* **2020**, *9*, doi:10.3390/pathogens9050386.

Eidesstaatliche Versicherung

Ich, Seyedeh Soraya Mousavi, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Untersuchungen zur antimikrobiellen und immun-modulierenden Wirksamkeit definierte Moleküle in *Campylobacter jejuni* infizierten Mäusen - Investigations on the antimicrobial and immune-modulating properties of defined molecules in *Campylobacter jejuni*-infected mice“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren/innen beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet.

Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Erstbetreuer/in, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

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Anteilserklärung an den erfolgten Publikationen

Seyedeh Soraya Mousavi hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1

Mousavi, Soraya, Ulrike Escher, Elisa Thunhorst, Sophie Kittler, Corinna Kehrenberg, Stefan Bereswill, and Markus M. Heimesaat. Vitamin C alleviates acute enterocolitis in *Campylobacter jejuni* infected mice. Scientific reports 10, no. 1 (2020): 1-13.

Beitrag im Einzelnen:

Versuchsplanung und -vorbereitung, Tierarbeit mit antibiotischer Behandlung, Rekolonisierung mit *Campylobacter jejuni*, Herstellung der Ascorbinsäure (Vitamin C) Lösung und Verabreichung, tägliche Tiervisite und Probenentnahme, Sektion, Aufarbeitung der Proben, mikrobiologische Auswertungen, Analysen der entnommenen Proben, statistische Aufarbeitung der Daten, Literaturrecherche, Verfassung des Manuskripts.

Publikation 2

Mousavi, Soraya, Anna-Maria Schmidt, Ulrike Escher, Sophie Kittler, Corinna Kehrenberg, Elisa Thunhorst, Stefan Bereswill, and Markus M. Heimesaat. Carvacrol ameliorates acute campylobacteriosis in a clinical murine infection model. Gut Pathogens 12, no. 1 (2020): 1-16.

Beitrag im Einzelnen:

Versuchsplanung und -vorbereitung, Tierarbeit mit antibiotischer Behandlung, Rekolonisierung mit *Campylobacter jejuni*, Herstellung der Carvacrol Lösung und Verabreichung, tägliche Tiervisite und Probenentnahme, Sektion, Aufarbeitung der Proben, mikrobiologische Auswertungen, Analysen der entnommenen Proben, statistische Aufarbeitung der Daten, Literaturrecherche, Verfassung des Manuskripts.

Publikation 3

Mousavi, Soraya, Fábia Daniela Lobo de Sá, Jörg-Dieter Schulzke, Roland Bücker, Stefan Bereswill, and Markus M. Heimesaat. Vitamin D in acute campylobacteriosis–results from an intervention study applying a clinical *Campylobacter jejuni* induced enterocolitis model. Frontiers in immunology 10 (2019): 2094.

Beitrag im Einzelnen:

Versuchsplanung und -vorbereitung, Tierarbeit mit antibiotischer Behandlung, Rekolonisierung mit *Campylobacter jejuni*, Herstellung der Cholecalciferol (Vitamin D) Lösung und Verabreichung, tägliche Tiervisite und Probenentnahme, Sektion, Aufarbeitung der Proben, mikrobiologische Auswertungen, Analysen der entnommenen Proben, statistische Aufarbeitung der Daten, Literaturrecherche, Verfassung des Manuskripts.

Unterschrift, Datum und Stempel des/des erstbetreuenden Hochschullehrers/in

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Unterschrift des Doktoranden/der Doktorandin

Druckexemplare der ausgewählten Publikationen (inklusive Journal Summary Listen)

Publikation 1

Soraya Mousavi, Ulrike Escher, Elisa Thunhorst, Sophie Kittler, Corinna Kehrenberg, Stefan Bereswill, and Markus M. Heimesaat. "Vitamin C alleviates acute enterocolitis in *Campylobacter jejuni* infected mice." Scientific reports 10, no. 1 (2020): 1-13.

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18	JOURNAL OF KING SAUD UNIVERSITY SCIENCE	1,640	3.819	0.002020
19	Journal of the Royal Society Interface	13,762	3.748	0.027670

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Vitamin C alleviates acute enterocolitis in *Campylobacter jejuni* infected mice

Soraya Mousavi[1], Ulrike Escher[1], Elisa Thunhorst[2], Sophie Kittler[2], Corinna Kehrenberg[3], Stefan Bereswill[1,4] & Markus M. Heimesaat[1,4*]

Human foodborne infections with the zoonotic pathogen *Campylobacter jejuni* are on the rise and constitute a significant socioeconomic burden worldwide. The health-beneficial, particularly anti-inflammatory effects of vitamin C (ascorbate) are well known. In our preclinical intervention study, we assessed potential anti-pathogenic and immunomodulatory effects of ascorbate in *C. jejuni*-infected secondary abiotic IL-10$^{-/-}$ mice developing acute campylobacteriosis similar to humans. Starting 4 days prior peroral *C. jejuni*-infection, mice received synthetic ascorbate via the drinking water until the end of the experiment. At day 6 post-infection, ascorbate-treated mice harbored slightly lower colonic pathogen loads and suffered from less severe *C. jejuni*-induced enterocolitis as compared to placebo control animals. Ascorbate treatment did not only alleviate macroscopic sequelae of infection, but also dampened apoptotic and inflammatory immune cell responses in the intestines that were accompanied by less pronounced pro-inflammatory cytokine secretion. Remarkably, the anti-inflammatory effects of ascorbate pretreatment in *C. jejuni*-infected mice were not restricted to the intestinal tract but could also be observed in extra-intestinal compartments including liver, kidneys and lungs. In conclusion, due to the potent anti-inflammatory effects observed in the clinical murine *C. jejuni*-infection model, ascorbate constitutes a promising novel option for prophylaxis and treatment of acute campylobacteriosis.

Campylobacter jejuni are the most common cause of food-borne gastroenteritis with increasing prevalence worldwide[1,2]. In fact, human campylobacteriosis represents a socioeconomic burden given estimated disease-associated costs of approximately 2.4 billion Euro[3]. Most commonly, *C. jejuni* transfer via consumption of contaminated raw or undercooked meat and milk or the ingestion of contaminated surface water to humans[4–8]. The intestinal colonization of *C. jejuni* induces a strong inflammatory response of the innate immune system affecting both, absorptive and secretory functions of the gastrointestinal tract[1]. In fact, campylobacteriosis constitutes a classical sodium malabsorption syndrome[9], which depending on the bacterial strain and the host immune status, results in illness of varying degree[10]. Whereas some patients remain asymptomatic or display mild symptoms, others develop fever, abdominal pain and watery diarrhea, or suffer from acute campylobacteriosis characterized by severe enterocolitis with inflammatory, bloody diarrhea[1,11]. In the majority of events, the disease is self-limited and treated symptomatically, whereas patients with immunosuppressive comorbidities require antibiotic treatment[11,12]. However, in few instances, post-infectious sequelae including Guillain-Barré syndrome, Miller Fisher syndrome, reactive arthritis and chronic inflammatory conditions of the intestinal tract might develop with a latent period of weeks or longer[1,13].

Even though human campylobacteriosis is becoming increasingly important, the distinct cellular and molecular mechanisms of host-pathogen interactions are limited. Clinical investigations in human patients disclosed that severe courses of *Campylobacter* infection and post-infectious morbidities (e.g., Guillain-Barré syndrome) are induced by the Gram-negative bacterial cell wall constituent lipooligosaccharide (LOS), especially sialylated LOS, which leads to hyper-activating of immune response[14]. Further RNA sequencing studies in human volunteers

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confirmed the major role of LOS-induced toll-like receptor (TLR) -4 signaling pathways in the induction of acute campylobacteriosis[9]. These findings support the view that campylobacteriosis results from a LOS-induced, TLR-4 mediated hyperergic innate immune response, which is similar to the inflammatory events induced by other LOS producing pathogens like *Neisseria meningitidis* and *N. gonorrhoeae*. However, *in vivo* trials have been hampered by the limited availability of experimental models. Mice show a strong physiological colonization resistance against invading microorganisms due to the mouse specific gut microbiota composition and are therefore protected from infection with enteropathogenic bacteria such as *C. jejuni*[15–17]. Furthermore, when compared to human, mice have been shown to be about 10,000-fold more resistant against LOS and lipopolysaccharide (LPS) expressed by Gram-negative bacteria[18–20]. We could recently show that a depletion of the gastrointestinal microbiota upon broad-spectrum antibiotic application in IL-10$^{-/-}$ mice facilitates intestinal *C. jejuni* colonization resulting in the development of key symptoms of acute human campylobacteriosis including wasting and bloody diarrhea within several days post-infection[21]. The main reasons for these severe *C. jejuni* induced immunopathological responses mounting in acute ulcerative enterocolitis are (i.) the abrogation of colonization resistance following microbiota depletion and (ii.) the lack of IL-10 enhancing susceptibility of mice to *C. jejuni* LOS[21]. In consequence, secondary abiotic IL-10$^{-/-}$ mice challenged with *C. jejuni* show strong intestinal and extra-intestinal immune responses via LOS–induced TLR-4 signaling[21–30]. Most importantly, the major role of LOS-induced intestinal immunopathology during campylobacteriosis was independently confirmed in elegant infection experiments with microbiota depleted SIGGR$^{-/-}$ mice developing campylobacteriosis similar to secondary abiotic IL-10 deficient mice. In contrast to the latter, the SIGGR$^{-/-}$ mice rendered sensitive to LOS due to the lack of a central inhibitor of cellular LPS/LOS-induced signaling pathways[31].

In the 1920s, vitamin C was first isolated by the Hungarian Nobel laureate Albert Szent Györgyi on track to unravel the options for treatment and prophylaxis of morbidities such as scurvy caused by deficiency of this (for humans essential) vitamin[32,33]. Patients suffering from scurvy exhibit poor wound healing due to weakening of collagenous structures and compromised immune cell functions[34] and are therefore highly susceptible to infections[32,35]. Ascorbate, the biologically active form of vitamin C, exerts a strong reductive potential and acts as a potent antioxidant that can be reversibly oxidized to dehydroascorbic acid[33,36]. Due to these characteristics, ascorbate is involved in several pivotal host defenses including immune regulatory pathways[35]. This is further underlined by the fact that both, innate and adaptive immune cells such as neutrophils, monocytes and lymphocytes, respectively, can accumulate ascorbate against a concentration gradient and exhibit intracellular concentrations that are up to 100 times higher than in plasma[36–38]. The presence of ascorbate in cells and plasma protects from oxidative stress. It is known that during phagocytosis, human granulocytes release hydrogen peroxide (H_2O_2) into the extracellular medium subsequently causing oxidative stress[39] and cell damage by lipid peroxidation and alteration of protein and nucleic acid structure[40]. Interestingly, due to this potent antioxidant property, ascorbate has been shown to neutralize H_2O_2 and to reduce the H_2O_2-induced apoptosis in periodontal tissues[41]. Moreover, endotoxin-induced oxidative stress due to reactive oxygen species (ROS) is associated with high cell mortality[42]. Increasing intracellular ascorbate concentrations, however, decrease ROS levels, thereby counteracting cell mortality[43].

Ascorbate has been shown to exert antimicrobial effects against distinct bacterial species such as *Mycobacterium tuberculosis*[44–47], *Staphylococcus aureus, Escherichia coli*[48], *Helicobacter pylori*[49,50] and *Salmonella* species[51] *in vitro*. Beside direct effects of ascorbate due to its low pH, for instance, reducing cell viability, it is also known that ascorbate exerts indirect bactericidal effects in the presence of metal ions or oxygen[52]. However, bacterial biofilm protects the cells against external influences and causes increased tolerance to antibiotic compounds[53]. Interestingly, ascorbate disrupts bacterial biofilm formation by inhibiting production of extracellular polymeric substances in *Bacillus subtilis*[54] and methicillin-resistant *S. aureus* (MRSA)[55] and pyocyanin production in *Pseudomonas aeruginosa*[56]. Thus, vitamin C (alone or in combination with antibiotics) constitutes a promising treatment option to destabilize bacterial biofilms.

Studies in the early 1980s revealed that growth of enteropathogens such as *C. jejuni* could be inhibited by ascorbate at low concentrations[52,57,58]. The authors have elegantly shown that the antimicrobial activity of ascorbate against *C. jejuni* is not due to lowered pH, but rather depends on the oxidation of ascorbate to dehydroascorbate and other products. However, the antimicrobial effects of ascorbate against *C. jejuni* have not been examined further and the exact mechanisms underlying the ascorbate-mediated toxicity against *C. jejuni* are still not known in detail. Moreover, data regarding potential immunomodulatory effects of ascorbate in *C. jejuni* infection are completely missing. This prompted us to perform for the first time a preclinical ascorbate intervention study applying our well-established murine clinical model of acute campylobacteriosis. Therefore, microbiota depleted IL-10$^{-/-}$ mice were subjected to synthetic ascorbate application via the drinking water, perorally infected with *C. jejuni* and surveyed for gastrointestinal pathogen loads, clinical outcome and intestinal as well as extra-intestinal immune responses during campylobacteriosis.

Results

Ascorbate treatment and gastrointestinal pathogen loads in *C. jejuni* infected secondary abiotic IL-10$^{-/-}$ mice. We first assessed antimicrobial effects of synthetic ascorbate against *C. jejuni in vitro*. Studies on minimal inhibitory concentrations (MICs) of 20 different *C. jejuni* isolates revealed a MIC_{90} value of 2818 mg/L (16 mM) with MICs ranging between 352 and 2818 mg/L (Supplementary Fig. S1). A MIC of 1409 mg/L (8 mM) was determined for the *C. jejuni* infection strain 81–176.

Secondary abiotic IL-10$^{-/-}$ mice were subjected to synthetic ascorbate treatment via the drinking water starting four days prior *C. jejuni* infection and lasting until necropsy. The concentration of the applied ascorbate solution was 5 g/L and hence, 3.56 times the MIC of the *C. jejuni* infection strain 81–176. On days 0 and 1, mice were then perorally challenged with 10^9 viable *C. jejuni* bacteria by gavage and surveyed until day 6 post-infection (p.i.). Bacterial culture analysis of faecal samples over time revealed that ascorbate treatment did

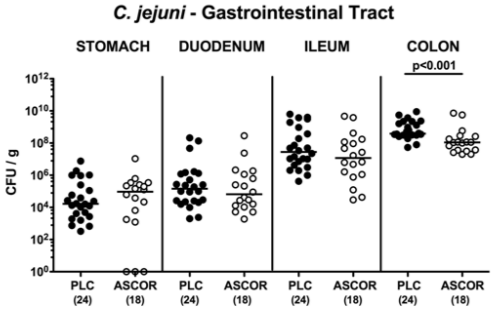

Figure 1. Gastrointestinal pathogen loads following ascorbate treatment of *C. jejuni* infected secondary abiotic IL-10$^{-/-}$ mice. Starting four days before peroral *C. jejuni* infection on days 0 and 1, secondary abiotic IL-10$^{-/-}$ mice were treated with synthetic ascorbate (ASCOR; open circles) or placebo (PLC; filled circles) via the drinking water. At day 6 post-infection, *C. jejuni* were isolated from distinct luminal parts of the gastrointestinal tract by culture and pathogenic loads expressed as colony forming units per gram (CFU/g). Medians, significance levels (p-values) assessed by the Mann-Whitney U test (for pairwise comparisons of PLC vs ASCOR in respective gastrointestinal compartment) and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments.

not affect intestinal colonization properties of the applied *C. jejuni* strain as indicated by comparable median loads of approximately 10^9 colony forming units per gram (CFU/g) derived from ascorbate or placebo (PLC) treated mice (n.s.; Supplementary Fig. S2). At day 6 p.i., colonic *C. jejuni* loads were slightly lower in ascorbate as compared to placebo treated mice (i.e., less than one log order of magnitude; $p < 0.001$; Fig. 1), whereas pathogen numbers were comparable in more proximal parts of the gastrointestinal tract including stomach, duodenum and ileum (n.s.; Fig. 1). Hence, ascorbate does only marginally affect intestinal *C. jejuni* colonization *in vivo*.

Ascorbate treatment ameliorates the clinical outcome of campylobacteriosis in *C. jejuni* infected secondary abiotic IL-10$^{-/-}$ mice. A kinetic survey of the clinical conditions of ascorbate versus PLC treated mice revealed that as early as day 5 p.i., the former suffered less distinctly from *C. jejuni* infection as compared to the latter (Supplementary Fig. S3). Upon necropsy (i.e., at day 6 p.i.), placebo treated mice were suffering from wasting and severe bloody diarrhea indicative for acute enterocolitis, whereas ascorbate treatment resulted in significantly reduced *C. jejuni* induced symptoms (Fig. 2A; Supplementary Fig. S3), particularly in less severe wasting, less pronounced diarrhea, less frequent abundance of blood in faecal samples and in better overall clinical appearance ($p < 0.05$–0.001 vs PLC; Fig. 2). Remarkably, almost 40% of infected mice from the ascorbate cohort were clinically uncompromised as indicated by cumulative clinical scores of 0 in 7 out of 18 cases ($p < 0.001$ vs PLC; Fig. 2A; Supplementary Fig. S3). Hence, ascorbate treatment alleviates clinical symptoms of campylobacteriosis.

Ascorbate reduces apoptosis in the colon of *C. jejuni* infected secondary abiotic IL-10$^{-/-}$ mice. We next quantitatively assessed pathogen-induced histopathological changes in hematoxylin and eosin (H&E) stained colonic paraffin sections applying a standardized histopathological scoring system[59]. At day 6 p.i., ascorbate treated mice displayed a trend towards slightly lower histopathological scores as compared to PLC control animal (n.s.; Fig. 3A).

Since apoptosis is regarded as a reliable parameter for the histopathological grading of intestinal inflammation[15], we further quantitatively determined apoptotic colonic epithelial cell numbers following *in situ* immunohistochemical staining of colonic paraffin sections with a caspase3 antibody. In fact, *C. jejuni* infection was associated with multifold increases in colonic apoptotic epithelial cells at day 6 p.i. ($p < 0.001$ vs naive, whereas this increase was far less pronounced in ascorbate as compared to PLC treated mice ($p < 0.001$; Fig. 3B; Supplementary Fig. S4). Hence, ascorbate treatment decreases *C. jejuni* induced apoptotic responses in colonic epithelial cells.

Less distinct pro-inflammatory immune cell responses in the colon following ascorbate treatment of *C. jejuni* infected secondary abiotic IL-10$^{-/-}$ mice. We next assessed large intestinal immune cell responses following ascorbate treatment of *C. jejuni* infected mice. *C. jejuni* infection was associated with marked increases of both, innate immune cell populations such as macrophages and monocytes ($p < 0.001$; Fig. 4A; Supplementary Fig. S5a) and adaptive immune cell subsets including T and B lymphocytes ($p < 0.01$–0.001; Fig. 4B,C; Supplementary Fig. S5b,c). At day 6 p.i., however, ascorbate treated mice displayed lower numbers of macrophages/monocytes as well as of T and B lymphocytes in their colonic mucosa and lamina propria as compared to infected PLC control animals ($p < 0.01$–0.001; Fig. 4; Supplementary Fig. S5). Hence, ascorbate treatment does not only alleviate macroscopic disease and colonic epithelial apoptosis following *C. jejuni* infection, but also dampens immune cell responses in the infected large intestines.

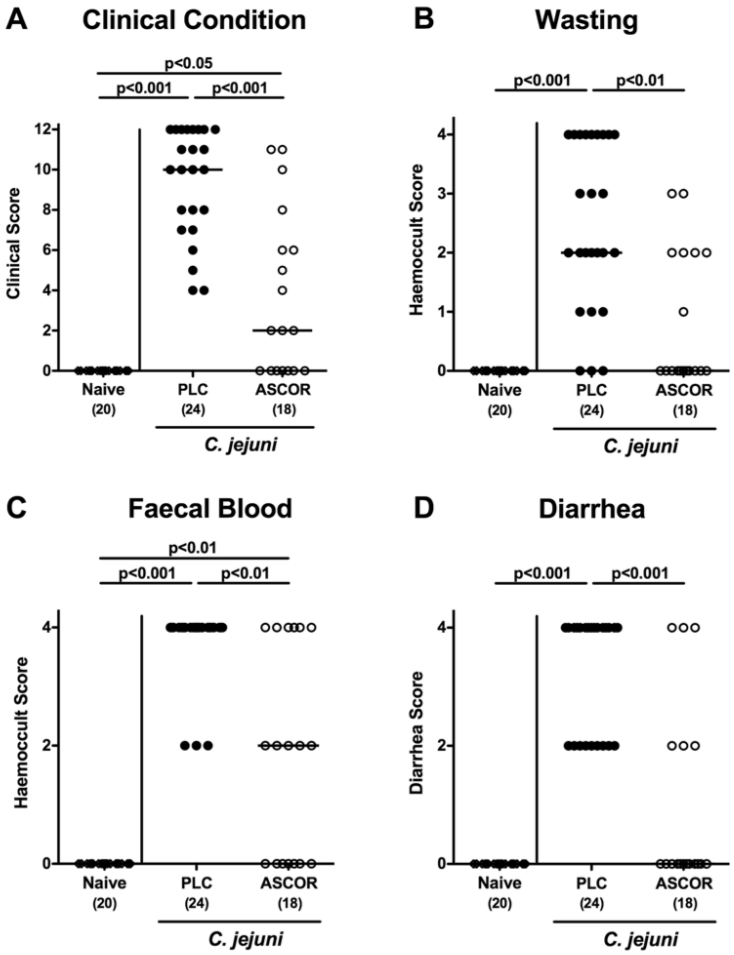

Figure 2. Macroscopic aspects of ascorbate treated mice following *C. jejuni* infection. Starting four days before peroral *C. jejuni* infection on days 0 and 1, secondary abiotic IL-$10^{-/-}$ mice were treated with synthetic ascorbate (ASCOR; open circles) or placebo (PLC; filled circles) via the drinking water. Applying a standardized clinical scoring system (see methods), **(A)** overall clinical outcome, **(B)** wasting, **(C)** abundance of faecal blood and **(D)** stool consistency were quantitatively surveyed at day 6 post-infection. Naive mice (open diamonds) served as uninfected controls. Medians, significance levels (p-values) assessed by the Kruskal Wallis test followed by Dunns correction and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments.

Reduced pro-inflammatory mediator secretion in the intestinal tract following ascorbate treatment of *C. jejuni* infected secondary abiotic IL-$10^{-/-}$ mice. We further surveyed pro-inflammatory mediator responses in the intestines of ascorbate versus PLC treated *C. jejuni* infected mice. Six days following *C. jejuni* infection elevated nitric oxide (NO) and tumor necrosis factor (TNF) concentrations could be measured in colonic *ex vivo* biopsies ($p < 0.01$–0.001 vs naive; Fig. 5A,B), whereas ascorbate treated mice displayed lower mediator levels in their large intestines as compared to PLC control animals ($p < 0.05$; Fig. 5A,B). Remarkably, elevated IL-6 and interferon-γ (IFN-γ) concentrations were determined in colonic *ex vivo* biopsies at day 6 p.i. of PLC ($p < 0.01$ and $p < 0.001$ vs naive, respectively), but not ascorbate treated mice (Fig. 5C,D), which also held true for IFN-γ levels measured in mesenteric lymph nodes (MLN) at day 6 p.i. ($p < 0.001$; Fig. 5E). Hence, ascorbate treatment dampens pro-inflammatory mediator responses in the large intestines of *C. jejuni* infected mice.

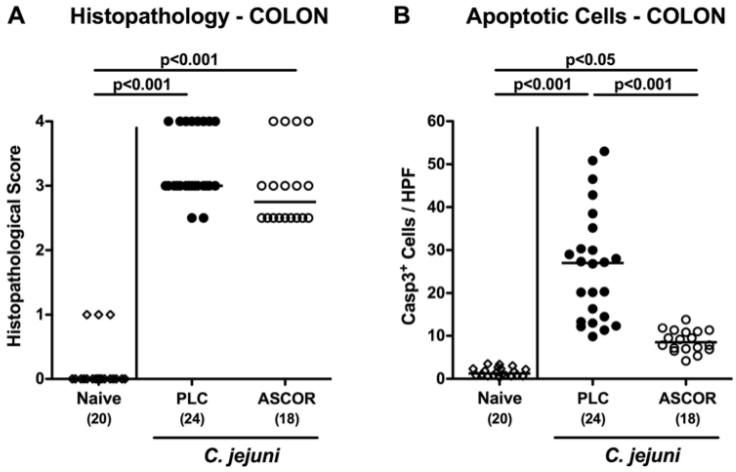

Figure 3. Histopathological and apoptotic cell responses in colonic epithelia of ascorbate treated mice following *C. jejuni* infection. Starting four days before peroral *C. jejuni* infection on days 0 and 1, secondary abiotic IL-10$^{-/-}$ mice were treated with synthetic ascorbate (ASCOR; open circles) or placebo (PLC; filled circles) via the drinking water. **(A)** Histopathological changes were quantitated in hematoxylin and eosin stained colonic paraffin sections applying a standardized scoring system as described in methods. **(B)** The average numbers of apoptotic (positive for caspase3, Casp3^{+}) colonic epithelial cells from six high power fields (HPF, 400x magnification) per mouse were assessed microscopically in immunohistochemically stained large intestinal paraffin sections at day 6 post-infection. Naive mice (open diamonds) served as uninfected and untreated controls. Medians, significance levels (p-values) assessed by the one-way ANOVA test followed by Tukey correction and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments.

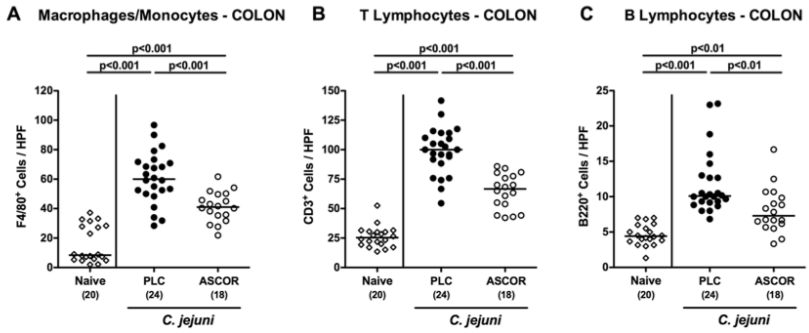

Figure 4. Colonic immune cell responses in ascorbate treated mice following *C. jejuni* infection. Starting four days before peroral *C. jejuni* infection on days 0 and 1, secondary abiotic IL-10$^{-/-}$ mice were treated with synthetic ascorbate (ASCOR; open circles) or placebo (PLC; filled circles) via the drinking water. The average numbers of **(A)** macrophages and monocytes (positive for F4/80), **(B)** T lymphocytes (positive for CD3), and **(C)** B lymphocytes (positive for B220) in the colonic mucosa and lamina propria from six high power fields (HPF, 400x magnification) per mouse were assessed microscopically in immunohistochemically stained large intestinal paraffin sections at day 6 post-infection. Naive mice (open diamonds) served as uninfected and untreated controls. Medians, significance levels (p-values) assessed by the one-way ANOVA test followed by Tukey correction and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments.

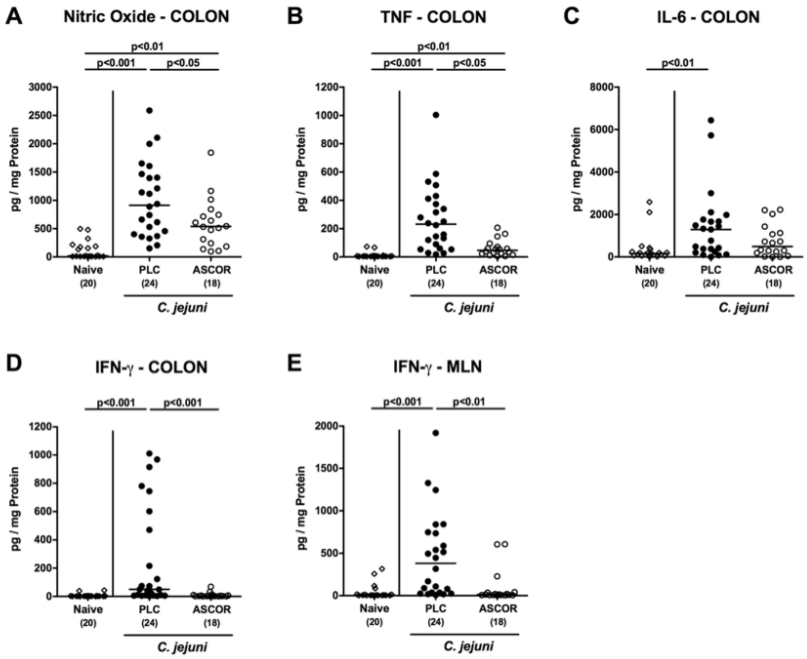

Figure 5. Intestinal inflammatory mediator responses in ascorbate treated mice following *C. jejuni* infection. Starting four days before peroral *C. jejuni* infection on days 0 and 1, secondary abiotic IL-10$^{-/-}$ mice were treated with synthetic ascorbate (ASCOR; open circles) or placebo (PLC; filled circles) via the drinking water. At day 6 post-infection. pro-inflammatory mediators including **(A)** nitric oxide, **(B)** TNF, **(C)** IL-6 and **(D)** IFN-γ were measured in supernatants of *ex vivo* biopsies derived from the colon as well as (**E**) IFN-γ concentrations assessed in mesenteric lymph nodes (MLN). Naive mice served as negative controls (open diamonds). Medians, significance levels (p-values) assessed by the Kruskall Wallis test followed by Dunns correction and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments.

Amelioration of inflammatory responses in extra-intestinal compartments following ascorbate treatment of *C. jejuni* infected secondary abiotic IL-10$^{-/-}$ mice. We further addressed whether the ascorbate associated disease-alleviating effects could also be observed in extra-intestinal compartments. We therefore quantitatively assessed apoptotic cell responses in kidneys, lungs and liver applying *in situ* immunohistochemistry. Six days following *C. jejuni* infection, increased numbers of caspase3^{+} cells could be determined in the kidneys and lungs of PLC ($p < 0.01$–0.001 vs naive), but not of ascorbate treated mice (Fig. 6A,B; Supplementary Fig. S6a,b). *C. jejuni* infected mice from either cohort displayed elevated hepatic apoptotic cell numbers ($p < 0.01$–0.001 vs naive), but with a trend towards lower numbers following ascorbate as compared to PLC application (n.s.; Fig. 6C; Supplementary Fig. S6c). Furthermore, PLC ($p < 0.001$ vs naive), but not ascorbate treated mice exhibited increased numbers of CD3^{+} T lymphocytes in their livers (Fig. 7A; Supplementary Fig. S6d), that was accompanied by elevated *C. jejuni* induced hepatic TNF secretion in the former, but not the latter cohort ($p < 0.05$ vs PLC; Fig. 7B).

We finally surveyed whether viable pathogens had translocated from the intestinal tract to extra-intestinal including systemic tissue sites following ascorbate treatment. *C. jejuni* translocation frequencies to MLN, spleen and liver were rather comparable following either treatment regimen at day 6 p.i. (Supplementary Fig. S7), whereas viable bacteria were isolated from the kidneys and lungs of PLC treated mice in single cases only, but in none of the animals from the ascorbate cohort (Supplementary Fig. S7). Of note, all blood samples taken at day 6 p.i. were *C. jejuni*-culture negative. Hence, the disease-alleviating effects of ascorbate treatment in *C. jejuni* infected mice were not restricted to the intestinal tract but could also be observed in extra-intestinal compartments.

Discussion

In this preclinical intervention study, we addressed for the first time potential anti-pathogenic and immunomodulatory properties of vitamin C during experimental acute campylobacteriosis in a clinical murine infection model. Following adding ascorbate to the drinking water starting four days prior *C. jejuni* infection of microbiota depleted IL-10$^{-/-}$ mice the excessively high gastrointestinal pathogen loads (of up to 10^9 viable bacterial cells per g faeces)

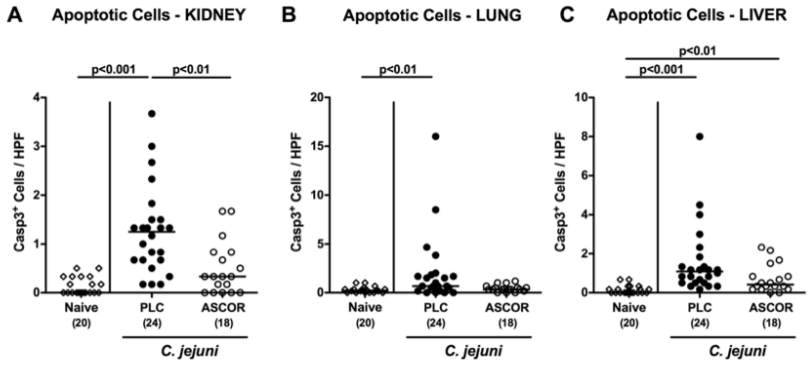

Figure 6. Extra-intestinal apoptosis in ascorbate treated mice following *C. jejuni* infection. Starting four days before peroral *C. jejuni* infection on days 0 and 1, secondary abiotic IL-10$^{-/-}$ mice were treated with synthetic ascorbate (ASCOR; open circles) or placebo (PLC; filled circles) via the drinking water. At day 6 post-infection, the average numbers of apoptotic cells (positive for caspase-3, Casp3^{+}) from six high power fields (HPF, 400x magnification) per mouse were assessed microscopically in immunohistochemically stained paraffin sections derived from **(A)** liver, **(B)** kidneys and **(C)** lungs. Naive mice served as uninfected and untreated controls (open diamonds). Medians, significance levels (p-values) assessed by the Kruskal Wallis test followed by Dunns correction and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments.

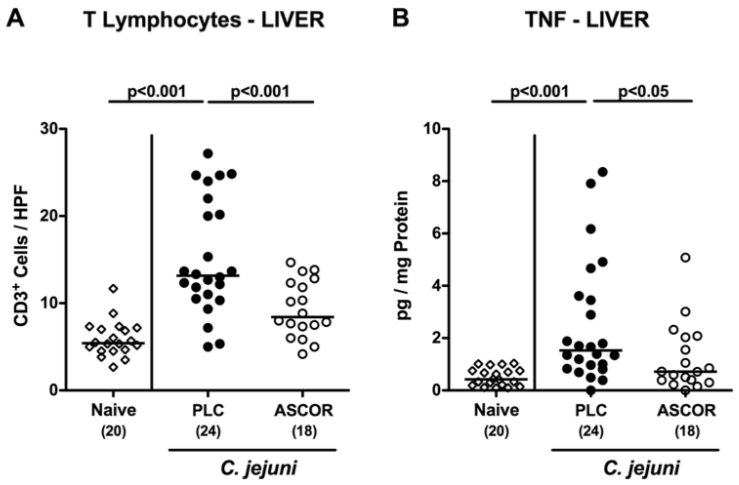

Figure 7. Hepatic inflammatory immune responses in ascorbate treated mice following *C. jejuni* infection. Starting four days before peroral *C. jejuni* infection on days 0 and 1, secondary abiotic IL-10$^{-/-}$ mice were treated with synthetic ascorbate (ASCOR; open circles) or placebo (PLC; filled circles) via the drinking water. At day 6 post-infection, the average numbers of **(A)** T lymphocytes (positive for CD3) from six high power fields (HPF, 400x magnification) per mouse were assessed microscopically in immunohistochemically stained paraffin sections derived from liver *ex vivo* biopsies. Furthermore, **(B)** TNF concentrations were determined in supernatants of hepatic *ex vivo* biopsies taken at day 6 p.i. Naive mice served as negative controls (open circles). Numbers of mice (in parentheses), medians (black bars) and significance levels (p-values) determined by the one-way ANOVA test followed by Tukey correction are indicated. Data shown were pooled from three independent experiments.

within the stably infected animals could only marginally be lowered by less than one order of magnitude until day 6 p.i. This result was not surprising given that the ascorbate concentration within the drinking water exceeded approximately 3.5 times the MIC against the applied *C. jejuni* strain but is reduced by mixing with the secretory body fluids in the intestinal tract of the animals. The potent antibacterial properties of ascorbate directed against food-borne pathogens such as *C. jejuni*[52,57,58] and *Salmonella* species[51,60,61] have been further supported by several previous *in vitro* studies. Initially, it was hypothesized that the antimicrobial effects of vitamin C were particularly due to its pH lowering properties[62]. But this is not the case for the antimicrobial activity of ascorbate against *C. jejuni*, which depends on oxidation of ascorbate to dehydroascorbic acid and other products, as confirmed in independent studies *in vitro*[52,58].

Despite the high gastrointestinal pathogen burdens, ascorbate treatment resulted in much better overall clinical conditions of *C. jejuni* infected mice including less severe diarrhea and less frequent abundance of faecal blood. It is noteworthy that almost 40% of mice from the ascorbate cohort were clinically uncompromised whereas mice from the placebo group suffered from full-blown campylobacteriosis as indicated by wasting and bloody diarrhea. The better macroscopic outcome upon ascorbate pretreatment of *C. jejuni* infected mice was accompanied by less apoptotic colonic epithelial cell responses. In support, *Helicobacter pylori*-induced apoptosis in human gastric epithelial cells was shown to be dampened after ascorbate treatment[63], whereas another study revealed that intracellular accumulation of ascorbate could suppress apoptotic pathways in human monocytes *in vitro*[64]. Moreover, co-incubation with ascorbate promoted proliferative properties of human peripheral T lymphocytes[65,66].

In addition to exerting antimicrobial effects, vitamin C has been shown to exhibit potent immunomodulatory, particularly anti-inflammatory properties both, *in vitro* and *in vivo*[67–69]. In our present study, ascorbate treatment resulted in less pronounced *C. jejuni*-induced pro-inflammatory innate as well as adaptive immune responses as indicated by lower numbers of macrophages and monocytes as well as of T and B lymphocytes, respectively, within the inflamed colonic mucosa and lamina propria that were accompanied by less intestinal secretion of pro-inflammatory mediators including nitric oxide, TNF, and IFN-γ and IL-6 at day 6 p.i. In support, ascorbate co-incubation resulted in down-regulated TNF levels in human whole blood[70] and in splenic mouse T cell cultures[71], whereas decreased nitric oxide, TNF, IFN-γ, and IL-6 concentrations could be determined in *Staphylococcus aureus* infected murine peritoneal macrophages *in vitro*[40]. Furthermore, ascorbate application to healthy subjects enhanced natural killer cell activities, lymphocyte proliferation and chemotaxis[72,73], further underlining the potent immunomodulatory, anti-inflammatory effects of vitamin C[69].

Notably, the anti-inflammatory effects of ascorbate treatment in *C. jejuni* infected mice were not restricted to the intestinal tract, but could also be observed in extra-intestinal organs as indicated by less distinct apoptosis in liver, kidneys and lungs and lower T cell numbers and lower TNF concentrations in livers of ascorbate versus placebo treated mice. Interestingly, translocation frequencies of viable *C. jejuni* from the intestinal tract to the liver were rather comparable following either treatment regimen, whereas no viable bacteria at all could be isolated from the kidneys and lungs following ascorbate pretreatment and in single cases only in placebo control mice at day 6 p.i.

The here presented immunomodulatory properties of an externally applied vitamin during acute *C. jejuni* induced enterocolitis are further supported by our very recent study surveying the health beneficial (i.e, anti-inflammatory) effects of vitamin D in the same clinical murine campylobacteriosis model. Following pre-treatment with synthetic 25-OH cholecalciferol, secondary abiotic IL-10$^{-/-}$ mice (i.) harbored comparably high *C. jejuni* loads in their gastrointestinal tract alike placebo controls upon peroral infection, but (ii.) suffered less frequently from diarrhea in the midst of infection, displayed (iii.) less pathogen-induced apoptotic, but (iv.) more pronounced counter-regulatory regenerative colonic cell responses that were accompanied by (v.) less distinct recruitment of both, innate und adaptive immune cells to the infected intestines, and by (vi.) less secretion of pro-inflammatory mediators in the intestinal tract (i.e, colon, ileum und MLN) as well as in the liver. Furthermore, as opposed to placebo controls (vii.) vitamin D treated mice displayed an uncompromised colonic epithelial barrier function which was accompanied by (viii.) less distinct bacterial translocation from the inflamed gut to extra-intestinal compartments in the latter as compared to the former[74].

The pharmacokinetic properties of ascorbate in the vertebrate host are well studied[75–79]. Murine investigations on absorption, tissue distribution and retention of ascorbate[76–78] revealed that maximum ascorbate concentrations could be measured in liver and kidneys (alike in the urine of humans[75]) approximately three hours following peroral single dose application[76]. Whereas in the lungs, adrenal glands, skin, white fat and pancreas peak levels were detectable as early as 6 hours, in the spleen increasing ascorbate concentrations could still be assessed until 24 hours following application[76]. The risk of adverse effects and intoxication upon high-dose ingestion may be considered negligible in subjects with intact renal function, given that vitamin C is water-soluble and concentrations exceeding the daily demands will be excreted via the kidneys[80].Together, ascorbate application can be considered as safe and has been pharmaceutically approved for the treatment of gastrointestinal morbidities in humans[33,60,81]. Interestingly, several inflammatory morbidities within the gastrointestinal tract have been shown to be associated with reduced ascorbate plasma concentrations[82,83].

In conclusion, our pre-clinical intervention study provides strong evidence for the first time that ascorbate constitutes a promising compound exerting potent anti-inflammatory and hence, disease-alleviating effects in non-self-limiting acute campylobacteriosis. Furthermore, food supplementation with ascorbate might be a useful tool to enhance host defense mechanisms in livestock animals directed against enteropathogens including *C. jejuni* thereby lowering pathogenic loads or even preventing from pathogen acquisition. Further studies are needed, however, to unravel the underlying mechanism in more detail.

Material and Methods

Ethical statement. *In vivo* experiments were carried out according to the European Guidelines for animal welfare (2010/63/EU) following agreement by the commission for animal experiments headed by the "Landesamt für Gesundheit und Soziales" (LaGeSo, Berlin, registration number G0172/16 and G0247/16). Animal welfare was monitored twice a day.

Determination of minimal inhibitory concentrations of ascorbate. To determine the antimicrobial effect of synthetic ascorbate, 20 *C. jejuni* isolates including the reference strain 81–176 (used for infection of mice, see below) and the DSM 4688 strain (for quality control) were tested in three independent experiments for their minimal inhibitory concentration (MIC) by the broth microdilution and macrodilution method. Settings of inoculum density, growth medium and conditions as well as incubation time were applied following the recommendations of the Clinical and Laboratory Standards Institute (CLSI) given in the document VET01-Ed5[84]. Twofold serial dilutions ranging from 0.03–32.0 mmol/L (6–5636 µg/mL) for ascorbate were tested. Stock solutions were prepared in Mueller-Hinton broth (Oxoid, Germany) and adjusted to pH 7.3.

Generation of secondary abiotic/gnotobiotic mice. In the identical unit of the Forschungseinrichtungen für Experimentelle Medizin (FEM, Charité - University Medicine Berlin), IL10$^{-/-}$ mice (C57BL/6j background) were bred, raised and housed under specific pathogen free (SPF) conditions. Mice were kept under standard conditions (22–24 °C room temperature, 55 ± 15% humidity, 12 h light/12 h dark cycle) in cages including filter tops within an experimental semi-barrier (accessible only with lab coat, overshoes, caps and sterile gloves) and had free access to autoclaved standard chow (food pellets: ssniff R/M-H, V1534-300, Sniff, Soest, Germany) as well as to autoclaved drinking water (*ad libitum*).

The depletion of the murine commensal intestinal microbiota in order to abrogate the physiological colonization resistance and hence, to assure stable intestinal *C. jejuni* colonization was achieved by application of five different antibiotics to the mice[15,85]. Briefly, 3-week old mice were treated with an antibiotic cocktail containing vancomycin (500 mg/L; Cell Pharm, Germany), ciprofloxacin (200 mg/L; Bayer Vital, Germany), imipenem (250 mg/L; MSD, Germany), metronidazole (1 g/L; Fresenius, Germany), and ampicillin plus sulbactam (1 g/L; Ratiopharm, Germany) within autoclaved drinking water (*ad libitum*) over a period time of 8 weeks. Three days prior infection the antibiotic treatment was withdrawn to assure antibiotic washout.

Treatment with synthetic ascorbate. Starting four days before *C. jejuni* infection and lasting until the end of the experiment, three-month old, sex-matched secondary abiotic IL-10$^{-/-}$ mice (maximum of three animals per cage) were treated with ascorbate (Sigma Aldrich, München, Germany) that had been sterile-filtered and added to the autoclaved tap water (*ad libitum*) and changed every other day. For ascorbate treatment, a daily dosage of 1 g per kg body weight was calculated[86]. Considering a body weight of approximately 25 g per mouse and a daily drinking volume of approximately 5 mL, the final concentration of the ascorbate solution was 5 g/L (pH 7.0). Mice from the placebo (PLC) cohort received autoclaved tap water only. Of note, the daily inter-individual drinking volumes between cages within and between respective cohorts were comparable. In four individual experiments, n = 5/5/4/4 ascorbate treated mice and n = 6/6/6/6 PLC controls were analyzed.

***C. jejuni* infection.** As previously described, mice were challenged with 10^9 CFU of the *C. jejuni* strain 81–176 (that had initially been isolated from a diseased patient suffering from bloody diarrhea) in a volume of 0.3 mL phosphate buffered saline (PBS; Gibco, life technologies, UK) on two successive days (days 0 and 1) by oral gavage[15]. In order to avoid contaminations, mice were kept and handled under strict aseptic conditions.

Evaluation of clinical conditions. The clinical conditions of mice were evaluated daily (starting four days before and lasting until day 6 after *C. jejuni* infection) and quantitated via standardized cumulative clinical scores (maximum 12 points), addressing the abundance of blood in faeces (0: no blood; 2: microscopic detection of blood by the Guajac method using Haemoccult, Beckman Coulter/PCD, Germany; 4: macroscopic blood visible), stool consistency (0: formed faeces; 2: pasty faeces; 4: liquid faeces), and the clinical aspect (0: normal; 2: ruffled fur, less locomotion; 4: isolation, severely compromised locomotion, pre-final aspect) as described earlier[27].

Sampling methods. Six days after the infection, mice were sacrificed by inhalation of isoflurane (Abbott, Germany). Luminal gastrointestinal samples (i.e., from colon, ileum, duodenum, and stomach) and *ex vivo* biopsies from intestinal (colon, ileum, MLN) and extra-intestinal (liver, kidneys and lungs) compartments were taken under aseptic conditions. To generate individual serum probes, cardiac blood was collected (approximately 1.0 mL). Intestinal samples for microbiological, immunological and immunohistopathological assays were taken in parallel from each mouse.

Histopathology. For histopathological analyses, sections (thickness 5 µm) of formalin-fixed (5%) and paraffin-embedded colonic *ex vivo* biopsies were used, stained with hematoxylin and eosin (H&E), and examined by light microscopy (100x magnification). The histopathological changes in the large intestines were quantitatively evaluated following an established histopathological scoring system ranging from 0 to 4 as described earlier[59]. Score 1: intact epithelium with minimal inflammatory cell infiltrates in the mucosa. Score 2: mild hyperplasia and mild goblet cell loss with mild inflammatory cell infiltrates in the mucosa and submucosa. Score 3: moderate goblet cell loss with moderate inflammatory cell infiltrates in the mucosa. Score 4: marked goblet cell loss, multiple crypt abscesses and crypt loss with marked inflammatory cell infiltration into in the mucosa and submucosa.

Immunohistochemical assays. *In situ* immunohistochemical analyses were conducted as previously reported[87,88]. Briefly, paraffin sections (5 μm) derived from *ex vivo* biopsies of interest (i.e., colon, liver, kidneys, lungs) were stained with primary antibodies directed against cleaved caspase 3 (Asp175, Cell Signaling, Beverly, MA, USA, 1:200) to detect apoptotic epithelial cells; with F4/80 (# 14–4801, clone BM8, eBioscience, San Diego, CA, USA, 1:50) to detect macrophages/monocytes; with CD3 (#N1580, Dako, 1:10) to detect T lymphocytes; and furthermore, with B220 (No. 14-0452-81, eBioscience; 1:200) to detect B lymphocytes. The sections were incubated with the primary antibody for 30 min followed by another 30 min of incubation with the respective secondary antibody (for anti-caspase-3 and anti-CD3 staining: biotinylated donkey anti-rabbit antibody; for anti-F4/80 and anti-B220: biotinylated rabbit anti-rat antibody; all from Dianova, Hamburg, Germany). As detection system, the Streptavidin-Alkaline Phosphatase Kit (Dako) using Fast Red as chromogen was applied. To generate negative controls, the primary antibodies had been excluded. The examination of positively stained cells was undertaken by light microscopy (magnification 100x and 400x). The average number of respective positively stained cells for each mouse was calculated within at least six high power fields (HPF, 0.287 mm^2, 400x magnification) by a blinded independent investigator. An Axiolmager Z1 microscope was used for the generation of images, which were subsequently processed with the Axiovision software (Carl Zeiss MicroImaging, Jena, Germany).

The colonization and translocation of *C. jejuni*. The *C. jejuni* loads were quantitatively surveyed in faeces samples taken every days after infection, and further, upon necropsy in gastrointestinal luminal samples (taken from stomach, duodenum, ileum and colon) and in homogenized *ex vivo* biopsies derived from MLN, spleen, liver, kidneys and lungs as well as in cardiac blood by culture as stated earlier[15,89]. The detection limit of viable pathogens was ≈100 CFU per g. For the determination of cumulative translocation rates of viable *C. jejuni* into respective extra-intestinal compartments, the ratio of the sum of culture-positive mice and the total numbers of analyzed animals (in %) out of four experiments were calculated.

Measurements of intestinal and extra-intestinal pro-inflammatory mediators. Longitudinally sliced and in PBS washed colonic *ex vivo* biopsies (strips of approximately 1 cm^2) as well as *ex vivo* biopsies derived from liver (approximately 1 cm^3) and MLN (3–4 lymph nodes) were cultured for 18 h at 37 °C in 24-flat-bottom well culture plates (Nunc, Germany) containing 500 μL serum-free RPMI 1640 medium (Gibco, life technologies, UK) that was supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL; PAA Laboratories, Germany). Using the Mouse Inflammation Cytometric Bead Assay (CBA; BD Biosciences, Germany) the culture supernatants and serum samples were analyzed for TNF, IFN-γ and IL-6 on a BD FACS Canto II flow cytometer (BD Biosciences). The nitric oxide concentrations were determined by the Griess reaction[85,90]. The pro-inflammatory mediator levels were normalized to the protein concentrations measured in the supernatant of the respective organ homogenate[88].

Data analysis. Data from four independently performed experiments were pooled and analyzed. The Mann-Whitney test (GraphPad Prism v7, USA) was used for determination of medians and levels of significance for pairwise comparisons of not normally distributed data, whereas for multiple comparisons, the one-sided ANOVA with Tukey post-correction or the Kruskal-Wallis test with Dunn's post-correction were used. Two-sided probability (p) values ≤ 0.05 were considered significant.

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References

1. Backert, S., Tegtmeyer, N., Cróinín, T. Ó., Boehm, M. & Heimesaat, M. M. In *Campylobacter* (ed. Günter Klein) 1–25 (Academic Press, 2017).
2. Fitzgerald, C. Campylobacter. *Clin. laboratory Med.* **35**, 289–298, https://doi.org/10.1016/j.cll.2015.03.001 (2015).
3. Authority, E. F. S. & Prevention, E. C. F. D. & Control. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017. *EFSA. J.* **16**, e05500, https://doi.org/10.2903/j.efsa.2018.5500 (2018).
4. Bronowski, C., James, C. E. & Winstanley, C. Role of environmental survival in transmission of Campylobacter jejuni. *FEMS Microbiol. Lett.* **356**, 8–19, https://doi.org/10.1111/1574-6968.12488 (2014).
5. Kaakoush, N. O., Castano-Rodriguez, N., Mitchell, H. M. & Man, S. M. Global Epidemiology of Campylobacter Infection. *Clin. Microbiol. Rev.* **28**, 687–720, https://doi.org/10.1128/CMR.00006-15 (2015).
6. Lane, J. A., Mehra, R. K., Carrington, S. D. & Hickey, R. M. The food glycome: a source of protection against pathogen colonization in the gastrointestinal tract. *Int. J. Food Microbiol.* **142**, 1–13, https://doi.org/10.1016/j.ijfoodmicro.2010.05.027 (2010).
7. Hermans, D. *et al.* Poultry as a host for the zoonotic pathogen Campylobacter jejuni. *Vector Borne Zoonotic Dis.* **12**, 89–98, https://doi.org/10.1089/vbz.2011.0676 (2012).
8. Newell, D., Mughini-Gras, L., Kalupahana, R. A. & Wagenaar, J. 85–110 (2016).
9. Bucker, R. *et al.* Campylobacter jejuni impairs sodium transport and epithelial barrier function via cytokine release in human colon. *Mucosal Immunol.* **11**, 575–577, https://doi.org/10.1038/mi.2017.78 (2018).
10. Poly, F. & Guerry, P. Pathogenesis of Campylobacter. *Curr. Opin. Gastroenterol.* **24**, 27–31, https://doi.org/10.1097/MOG.0b013e3282f1dcb1 (2008).
11. Kist, M. & Bereswill, S. Campylobacter jejuni. *Contrib. Microbiol.* **8**, 150–165 (2001).
12. Walker, R. I. *et al.* Pathophysiology of Campylobacter enteritis. *Microbiol. Rev.* **50**, 81–94 (1986).
13. Keithlin, J., Sargeant, J., Thomas, M. K. & Fazil, A. Systematic review and meta-analysis of the proportion of Campylobacter cases that develop chronic sequelae. *BMC Public. Health* **14**, 1203, https://doi.org/10.1186/1471-2458-14-1203 (2014).
14. Mortensen, N. P. *et al.* Sialylation of Campylobacter jejuni lipo-oligosaccharides is associated with severe gastro-enteritis and reactive arthritis. *Microbes Infect.* **11**, 988–994, https://doi.org/10.1016/j.micinf.2009.07.004 (2009).
15. Bereswill, S. *et al.* Novel murine infection models provide deep insights into the "menage a trois" of Campylobacter jejuni, microbiota and host innate immunity. *Plos One* **6**, e20953, https://doi.org/10.1371/journal.pone.0020953 (2011).
16. Masanta, W. O. *et al.* Modification of intestinal microbiota and its consequences for innate immune response in the pathogenesis of campylobacteriosis. *Clin. Dev. Immunol.* **2013**, 526860, https://doi.org/10.1155/2013/526860 (2013).

17. Fiebiger, U., Bereswill, S. & Heimesaat, M. M. Dissecting the Interplay Between Intestinal Microbiota and Host Immunity in Health and Disease: Lessons Learned from Germfree and Gnotobiotic Animal Models. *Eur. J. Microbiol. Immunol.* **6**, 253–271, https://doi.org/10.1556/1886.2016.00036 (2016).
18. Warren, H. S. *et al.* Resilience to bacterial infection: difference between species could be due to proteins in serum. *J. Infect. Dis.* **201**, 223–232, https://doi.org/10.1086/649557 (2010).
19. Robertson, S. A., Care, A. S. & Skinner, R. J. Interleukin 10 regulates inflammatory cytokine synthesis to protect against lipopolysaccharide-induced abortion and fetal growth restriction in mice. *Biol. Reprod.* **76**, 738–748, https://doi.org/10.1095/biolreprod.106.056143 (2007).
20. da Silva, A. M. T. *et al.* Shock and multiple-organ dysfunction after self-administration of Salmonella endotoxin. *N. Engl. J. Med.* **328**, 1457–1460 (1993).
21. Haag, L. M. *et al.* Campylobacter jejuni induces acute enterocolitis in gnotobiotic IL-$10^{-/-}$ mice via Toll-like-receptor-2 and -4 signaling. *PLoS One* **7**, e40761, https://doi.org/10.1371/journal.pone.0040761 (2012).
22. Haag, L. M. *et al.* Campylobacter jejuni infection of infant mice: acute enterocolitis is followed by asymptomatic intestinal and extra-intestinal immune responses. *Eur. J. Microbiol. Immunol.* **2**, 2–11, https://doi.org/10.1556/EuJMI.2.2012.1.2 (2012).
23. Alutis, M. E. *et al.* Selective gelatinase inhibition reduces apoptosis and pro-inflammatory immune cell responses in Campylobacter jejuni-infected gnotobiotic IL-10 deficient mice. *Eur. J. Microbiol. Immunol.* **4**, 213–222, https://doi.org/10.1556/EUJMI-D-14-00031 (2014).
24. Bereswill, S. *et al.* Lactobacillus johnsonii ameliorates intestinal, extra-intestinal and systemic pro-inflammatory immune responses following murine Campylobacter jejuni infection. *Sci. Rep.* **7**, 2138, https://doi.org/10.1038/s41598-017-02436-2 (2017).
25. Bereswill, S., Grundmann, U., Alutis, M. E., Fischer, A. & Heimesaat, M. M. Campylobacter jejuni infection of conventionally colonized mice lacking nucleotide-oligomerization-domain-2. *Gut Pathog.* **9**, 5, https://doi.org/10.1186/s13099-017-0155-3 (2017).
26. Ekmekciu, I., Fiebiger, U., Stingl, K., Bereswill, S. & Heimesaat, M. M. Amelioration of intestinal and systemic sequelae of murine Campylobacter jejuni infection by probiotic VSL#3 treatment. *Gut Pathog.* **9**, 17, https://doi.org/10.1186/s13099-017-0168-y (2017).
27. Heimesaat, M. M. *et al.* The role of serine protease HtrA in acute ulcerative enterocolitis and extra-intestinal immune responses during Campylobacter jejuni infection of gnotobiotic IL-10 deficient mice. *Front. Cell Infect. Microbiol.* **4**, 77, https://doi.org/10.3389/fcimb.2014.00077 (2014).
28. Heimesaat, M. M., Grundmann, U., Alutis, M. E., Fischer, A. & Bereswill, S. Microbiota Composition and Immune Responses During Campylobacter Jejuni Infection in Conventionally Colonized IL-10(−/−) Mice Lacking Nucleotide Oligomerization Domain 2. *Eur. J. Microbiol. Immunol.* **7**, 1–14, https://doi.org/10.1556/1886.2016.00038 (2017).
29. Heimesaat, M. M., Grundmann, U., Alutis, M. E., Fischer, A. & Bereswill, S. Absence of Nucleotide-Oligomerization-Domain-2 Is Associated with Less Distinct Disease in Campylobacter jejuni Infected Secondary Abiotic IL-10 Deficient Mice. *Front. Cell Infect. Microbiol.* **7**, 322, https://doi.org/10.3389/fcimb.2017.00322 (2017).
30. Schmidt, A. M. *et al.* Protease Activity of Campylobacter jejuni HtrA Modulates Distinct Intestinal and Systemic Immune Responses in Infected Secondary Abiotic IL-10 Deficient Mice. *Front. Cell Infect. Microbiol.* **9**, 79, https://doi.org/10.3389/fcimb.2019.00079 (2019).
31. Stahl, M. *et al.* A novel mouse model of Campylobacter jejuni gastroenteritis reveals key pro-inflammatory and tissue protective roles for Toll-like receptor signaling during infection. *Plos Pathog.* **10**, e1004264 (2014).
32. Hemilä, H. Vitamin C and Infections. *Nutrients* **9**, 339, https://doi.org/10.3390/nu9040339 (2017).
33. Sorice, A. *et al.* Ascorbic acid: its role in immune system and chronic inflammation diseases. *Mini Rev. Med. Chem.* **14**, 444–452 (2014).
34. Ang, A., Pullar, J. M., Currie, M. J. & Vissers, M. C. Vitamin C and immune cell function in inflammation and cancer. *Biochemical Soc. Trans.* **46**, 1147–1159 (2018).
35. Carr, A. C. & Maggini, S. Vitamin C and Immune Function. *Nutrients* **9**, https://doi.org/10.3390/nu9111211 (2017).
36. Strohle, A., Wolters, M. & Hahn, A. Micronutrients at the interface between inflammation and infection–ascorbic acid and calciferol: part 1, general overview with a focus on ascorbic acid. *Inflamm. Allergy Drug. Targets* **10**, 54–63 (2011).
37. Washko, P., Rotrosen, D. & Levine, M. Ascorbic acid in human neutrophils. *Am. J. Clin. Nutr.* **54**, 1221S–1227S, https://doi.org/10.1093/ajcn/54.6.1221s (1991).
38. Levine, M., Wang, Y., Padayatty, S. J. & Morrow, J. A new recommended dietary allowance of vitamin C for healthy young women. *Proc. Natl Acad. Sci. USA* **98**, 9842–9846, https://doi.org/10.1073/pnas.171318198 (2001).
39. Root, R. K. & Metcalf, J. A. H_2O_2 release from human granulocytes during phagocytosis. Relationship to superoxide anion formation and cellular catabolism of H_2O_2: studies with normal and cytochalasin B-treated cells. *J. Clin. investigation* **60**, 1266–1279, https://doi.org/10.1172/jci108886 (1977).
40. Dey, S. & Bishayi, B. Killing of S. aureus in murine peritoneal macrophages by Ascorbic acid along with antibiotics Chloramphenicol or Ofloxacin: Correlation with inflammation. *Microb. Pathog.* **115**, 239–250, https://doi.org/10.1016/j.micpath.2017.12.048 (2018).
41. Wu, W. *et al.* Effect of vitamin C administration on hydrogen peroxide-induced cytotoxicity in periodontal ligament cells. *Mol. Med. Rep.* **11**, 242–248, https://doi.org/10.3892/mmr.2014.2712 (2015).
42. Baxter, F. Septic Shock. *Can. J. Anaesth.* **44**, 59–72, https://doi.org/10.1007/bf03014326 (1997).
43. Qiao, H. & May, J. M. Macrophage differentiation increases expression of the ascorbate transporter (SVCT2). *Free. Radic. Biol. Med.* **46**, 1221–1232, https://doi.org/10.1016/j.freeradbiomed.2009.02.004 (2009).
44. Boissevain, C. H. & Spillane, J. H. Jr. A note on the effect of synthetic ascorbic acid (vitamin C) on the growth of the tubercle bacillus. *Am. Rev. Tuberculosis* **35**, 661–662 (1937).
45. St, J. L. Der Einfluss von Vitamin C und Vitamin B 1 auf das Wachstum der Tuberkelbacillen. *Klinische Wochenschr.* **16**, 1423–1425 (1937).
46. Sirsi, M. Antimicrobial action of vitamin C on M. tuberculosis and some other pathogenic organisms. *Indian. J. Med. Sci.* **6**, 5 (1952).
47. Myrvik, Q. N. & Volk, W. A. Comparative study of the antibacterial properties of ascorbic acid and reductogenic compounds. *J. Bacteriol.* **68**, 622 (1954).
48. Kallio, J., Jaakkola, M., Maki, M., Kilpelainen, P. & Virtanen, V. Vitamin C inhibits staphylococcus aureus growth and enhances the inhibitory effect of quercetin on growth of Escherichia coli *in vitro*. *Planta Med.* **78**, 1824–1830, https://doi.org/10.1055/s-0032-1315388 (2012).
49. Zhang, Z. W. & Farthing, M. J. The roles of vitamin C in Helicobacter pylori associated gastric carcinogenesis. *Chin. J. Dig. Dis.* **6**, 53–58, https://doi.org/10.1111/j.1443-9573.2005.00194.x (2005).
50. Zhang, H. M., Wakisaka, N., Maeda, O. & Yamamoto, T. Vitamin C inhibits the growth of a bacterial risk factor for gastric carcinoma: Helicobacter pylori. *Cancer* **80**, 1897–1903 (1997).
51. Ghosh, T. *et al.* A Combination of Linalool, Vitamin C, and Copper Synergistically Triggers Reactive Oxygen Species and DNA Damage and Inhibits Salmonella enterica subsp. enterica Serovar Typhi and Vibrio fluvialis. *Appl Environ Microbiol* **85**, https://doi.org/10.1128/AEM.02487-18 (2019).
52. Juven, B. J. & Kanner, J. Effect of ascorbic, isoascorbic and dehydroascorbic acids on the growth and survival of Campylobacter jejuni. *J. Appl. Bacteriol.* **61**, 339–345 (1986).
53. Hoiby, N., Bjarnsholt, T., Givskov, M., Molin, S. & Ciofu, O. Antibiotic resistance of bacterial biofilms. *Int. J. antimicrobial agents* **35**, 322–332, https://doi.org/10.1016/j.ijantimicag.2009.12.011 (2010).

54. Pandit, S. *et al.* Low Concentrations of Vitamin C Reduce the Synthesis of Extracellular Polymers and Destabilize Bacterial Biofilms. *Frontiers in Microbiology* **8**, https://doi.org/10.3389/fmicb.2017.02599 (2017).
55. Ali Mirani, Z. *et al.* Ascorbic acid augments colony spreading by reducing biofilm formation of methicillin-resistant Staphylococcus aureus. *Iran. J. basic. Med. Sci.* **21**, 175–180, https://doi.org/10.22038/ijbms.2018.20714.5398 (2018).
56. El-Mowafy, S. A. & Shaaban, M. I. & Abd El Galil, K. H. Sodium ascorbate as a quorum sensing inhibitor of Pseudomonas aeruginosa. *J. Appl. Microbiology* **117**, 1388–1399, https://doi.org/10.1111/jam.12631 (2014).
57. Juven, B. J., Kanner, J., Weisslowicz, H. & Harel, S. Effect of Ascorbic and Isoascorbic acids on Survival of Campylobacter jejuni in Poultry Meat (1). *J. Food Prot.* **51**, 436–437, https://doi.org/10.4315/0362-028X-51.6.436 (1988).
58. Fletcher, R. D., Albers, A. C., Chen, A. K. & Albertson, J. N. Jr. Ascorbic acid inhibition of Campylobacter jejuni growth. *Appl. Env. Microbiol.* **45**, 792–795 (1983).
59. Erben, U. *et al.* A guide to histomorphological evaluation of intestinal inflammation in mouse models. *Int. J. Clin. Exp. Pathol.* **7**, 4557–4576 (2014).
60. Hernandez-Patlan, D. *et al.* Evaluation of the Dietary Supplementation of a Formulation Containing Ascorbic Acid and a Solid Dispersion of Curcumin with Boric Acid against Salmonella Enteritidis and Necrotic Enteritis in Broiler Chickens. *Animals (Basel)* **9**, https://doi.org/10.3390/ani9040184 (2019).
61. Hartzen, S. H., Frimodt-Moller, N. & Frolund Thomsen, V. The antibacterial activity of a siderophore. 1. *In vitro* activity of deferoxamine alone and in combination with ascorbic acid on Staphylococcus aureus. *APMIS: acta pathologica, microbiologica, et. immunologica Scandinavica* **97**, 419–424 (1989).
62. v. Gagyi, J. Ueber die bactericide und antitoxische Wirkung des Vitamin C. *J. Mol. Med.* **15**, 190–195 (1936).
63. Chen, X., Liu, R., Liu, X., Xu, C. & Wang, X. L-ascorbic Acid-2-Glucoside inhibits Helicobacter pylori-induced apoptosis through mitochondrial pathway in Gastric Epithelial cells. *Biomed. Pharmacother.* **97**, 75–81, https://doi.org/10.1016/j.biopha.2017.10.030 (2018).
64. Perez-Cruz, I., Carcamo, J. M. & Golde, D. W. Vitamin C inhibits FAS-induced apoptosis in monocytes and U937 cells. *Blood* **102**, 336–343, https://doi.org/10.1182/blood-2002-11-3559 (2003).
65. Huijskens, M. J. *et al.* Technical advance: ascorbic acid induces development of double-positive T cells from human hematopoietic stem cells in the absence of stromal cells. *J. Leukoc. Biol.* **96**, 1165–1175, https://doi.org/10.1189/jlb.1TA0214-121RR (2014).
66. Molina, N., Morandi, A. C., Bolin, A. P. & Otton, R. Comparative effect of fucoxanthin and vitamin C on oxidative and functional parameters of human lymphocytes. *Int. Immunopharmacol.* **22**, 41–50, https://doi.org/10.1016/j.intimp.2014.06.026 (2014).
67. Carcamo, J. M., Borquez-Ojeda, O. & Golde, D. W. Vitamin C inhibits granulocyte macrophage-colony-stimulating factor-induced signaling pathways. *Blood* **99**, 3205–3212, https://doi.org/10.1182/blood.v99.9.3205 (2002).
68. Bowie, A. G. & O'Neill, L. A. Vitamin C inhibits NF-kappa B activation by TNF via the activation of p38 mitogen-activated protein kinase. *J. Immunol.* **165**, 7180–7188, https://doi.org/10.4049/jimmunol.165.12.7180 (2000).
69. Mousavi, S., Bereswill, S. & Heimesaat, M. M. Immunomodulatory and Antimicrobial Effects of Vitamin C. *Eur. J. Microbiology Immunol.* https://doi.org/10.1556/1886.2019.00016 (2019).
70. Hartel, C., Strunk, T., Bucsky, P. & Schultz, C. Effects of vitamin C on intracytoplasmic cytokine production in human whole blood monocytes and lymphocytes. *Cytokine* **27**, 101–106, https://doi.org/10.1016/j.cyto.2004.02.004 (2004).
71. Maeng, H. G. *et al.* Vitamin C enters mouse T cells as dehydroascorbic acid *in vitro* and does not recapitulate *in vivo* vitamin C effects. *Immunobiology* **214**, 311–320, https://doi.org/10.1016/j.imbio.2008.09.003 (2009).
72. Levy, R., Shriker, O., Porath, A., Riesenberg, K. & Schlaeffer, F. Vitamin C for the treatment of recurrent furunculosis in patients with imparied neutrophil functions. *J. Infect. Dis.* **173**, 1502–1505, https://doi.org/10.1093/infdis/173.6.1502 (1996).
73. Heuser, G. & Vojdani, A. Enhancement of natural killer cell activity and T and B cell function by buffered vitamin C in patients exposed to toxic chemicals: the role of protein kinase-C. *Immunopharmacol. Immunotoxicol.* **19**, 291–312, https://doi.org/10.3109/08923979709046977 (1997).
74. Mousavi, S. *et al.* Vitamin D in Acute Campylobacteriosis-Results From an Intervention Study Applying a Clinical Campylobacter jejuni Induced Enterocolitis Model. *Front. immunology* **10**, 2094, https://doi.org/10.3389/fimmu.2019.02094 (2019).
75. Lykkesfeldt, J. & Tveden-Nyborg, P. The Pharmacokinetics of Vitamin C. *Nutrients* **11**, 2412, https://doi.org/10.3390/nu11102412 (2019).
76. Iwama, M., Shimokado, K., Maruyama, N. & Ishigami, A. Time course of vitamin C distribution and absorption after oral administration in SMP30/GNL knockout mice. *Nutr.* **27**, 471–478, https://doi.org/10.1016/j.nut.2010.04.010 (2011).
77. Aumailley, L. *et al.* Vitamin C modulates the metabolic and cytokine profiles, alleviates hepatic endoplasmic reticulum stress, and increases the life span of Gulo−/− mice. *Aging* **8**, 458–483, https://doi.org/10.18632/aging.100902 (2016).
78. Vissers, M. C., Bozonet, S. M., Pearson, J. F. & Braithwaite, L. J. Dietary ascorbate intake affects steady state tissue concentrations in vitamin C-deficient mice: tissue deficiency after suboptimal intake and superior bioavailability from a food source (kiwifruit). *Am. J. Clin. Nutr.* **93**, 292–301, https://doi.org/10.3945/ajcn.110.004853 (2011).
79. Tsukaguchi, H. *et al.* A family of mammalian Na+-dependent L-ascorbic acid transporters. *Nat.* **399**, 70–75, https://doi.org/10.1038/19986 (1999).
80. Chambial, S., Dwivedi, S., Shukla, K. K., John, P. J. & Sharma, P. Vitamin C in disease prevention and cure: an overview. *Indian. J. Clin. Biochem.* **28**, 314–328, https://doi.org/10.1007/s12291-013-0375-3 (2013).
81. Aditi, A. & Graham, D. Y. Vitamin C, gastritis, and gastric disease: a historical review and update. *Digestive Dis. Sci.* **57**, 2504–2515, https://doi.org/10.1007/s10620-012-2203-7 (2012).
82. Ichim, T. E. *et al.* Intravenous ascorbic acid to prevent and treat cancer-associated sepsis? *J. Transl. Med.* **9**, 25, https://doi.org/10.1186/1479-5876-9-25 (2011).
83. El Halabi, I. *et al.* Ascorbic Acid in Colon Cancer: From the Basic to the Clinical Applications. *International journal of molecular sciences* **19**, https://doi.org/10.3390/ijms19092752 (2018).
84. CLSI. VET01-A5: Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standard. 5th Edition. Clinical and Laboratory Standards Institute, Wayne, PA. Volume 34 (2018).
85. Heimesaat, M. M. *et al.* Gram-negative bacteria aggravate murine small intestinal Th1-type immunopathology following oral infection with Toxoplasma gondii. *J. Immunol.* **177**, 8785–8795 (2006).
86. Kockar, C., Ozturk, M. & Bavbek, N. Helicobacter pylori eradication with beta carotene, ascorbic acid and allicin. *Acta Medica* **44**, 97–100 (2001).
87. Heimesaat, M. M. *et al.* Impact of Campylobacter jejuni cj0268c knockout mutation on intestinal colonization, translocation, and induction of immunopathology in gnotobiotic IL-10 deficient mice. *Plos One* **9**, e90148, https://doi.org/10.1371/journal.pone.0090148 (2014).
88. Heimesaat, M. M., Giladi, E., Kuhl, A. A., Bereswill, S. & Gozes, I. The octapetide NAP alleviates intestinal and extra-intestinal anti-inflammatory sequelae of acute experimental colitis. *Peptides* **101**, 1–9, https://doi.org/10.1016/j.peptides.2017.12.023 (2018).
89. Heimesaat, M. M. *et al.* Survey of extra-intestinal immune responses in asymptomatic long-term Campylobacter jejuni-infected mice. *Eur. J. Microbiol. Immunol.* **3**, 174–182, https://doi.org/10.1556/EuJMI.3.2013.3.4 (2013).
90. Bryan, N. S. & Grisham, M. B. Methods to detect nitric oxide and its metabolites in biological samples. *Free. Radic. Biol. Med.* **43**, 645–657, https://doi.org/10.1016/j.freeradbiomed.2007.04.026 (2007).

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Author contributions

S.M.: Performed experiments, co-wrote paper. U.E.: Performed experiments, co-edited paper. E.T.: Performed experiments, analyzed data, co-edited paper. S.K.: Performed experiments, analyzed data, co-edited paper. C.K.: Performed experiments, analyzed data, co-edited paper. S.B.: Provided advice in experimental design, critically discussed results, co-edited paper. M.M.H.: Designed and performed experiments, analyzed data, wrote paper.

Competing interests

The authors declare no competing interests.

Additional information

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Publikation 2

Soraya Mousavi, Anna-Maria Schmidt, Ulrike Escher, Sophie Kittler, Corinna Kehrenberg, Elisa Thunhorst, Stefan Bereswill, and Markus M. Heimesaat. "Carvacrol ameliorates acute campylobacteriosis in a clinical murine infection model." Gut Pathogens 12, no. 1 (2020): 1-16.

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8	Microbiome	7,321	11.607	0.028720
9	Annual Review of Microbiology	10,132	11.000	0.009490
10	ISME Journal	26,474	9.180	0.051810
...				
54	Advances in Applied Microbiology	1,789	3.343	0.001570
55	Gut Pathogens	1,399	3.274	0.002960
56	SYSTEMATIC AND APPLIED MICROBIOLOGY	4,965	3.224	0.004360

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Gut Pathogens

RESEARCH Open Access

Carvacrol ameliorates acute campylobacteriosis in a clinical murine infection model

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Abstract

Background: The prevalence of human infections with the zoonotic pathogen *Campylobacter jejuni* is rising worldwide. Therefore, the identification of compounds with potent anti-pathogenic and anti-inflammatory properties for future therapeutic and/or preventive application to combat campylobacteriosis is of importance for global health. Results of recent studies suggested carvacrol (4-isopropyl-2-methylphenol) as potential candidate molecule for the treatment of campylobacteriosis in humans and for the prevention of *Campylobacter* colonization in farm animals.

Results: To address this in a clinical murine infection model of acute campylobacteriosis, secondary abiotic IL-10$^{-/-}$ mice were subjected to synthetic carvacrol via the drinking water starting 4 days before peroral *C. jejuni* challenge. Whereas at day 6 post-infection placebo treated mice suffered from acute enterocolitis, mice from the carvacrol cohort not only harbored two log orders of magnitude lower pathogen loads in their intestines, but also displayed significantly reduced disease symptoms. Alleviated campylobacteriosis following carvacrol application was accompanied by less distinct intestinal apoptosis and pro-inflammatory immune responses as well as by higher numbers of proliferating colonic epithelial cells. Remarkably, the inflammation-ameliorating effects of carvacrol treatment were not restricted to the intestinal tract, but could also be observed in extra-intestinal organs such as liver, kidneys and lungs and, strikingly, systemically as indicated by lower IFN-γ, TNF, MCP-1 and IL-6 serum concentrations in carvacrol versus placebo treated mice. Furthermore, carvacrol treatment was associated with less frequent translocation of viable *C. jejuni* originating from the intestines to extra-intestinal compartments.

Conclusion: The lowered *C. jejuni* loads and alleviated symptoms observed in the here applied clinical murine model for human campylobacteriosis highlight the application of carvacrol as a promising novel option for both, the treatment of campylobacteriosis and hence, for prevention of post-infectious sequelae in humans, and for the reduction of *C. jejuni* colonization in the intestines of vertebrate lifestock animals.

Keywords: Carvacrol, Anti-pathogenic and anti-inflammatory properties, *Campylobacter jejuni*, Secondary abiotic IL-10$^{-/-}$ mice, Pro-inflammatory immune responses, Bacterial translocation, Host–pathogen-interaction, Intestinal immunopathology, Extra-intestinal and systemic immune responses

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Background

In the United States, up to 10 million foodborne-related cases were estimated to be responsible for approximately 1300 deaths annually [1]. The presence of distinct bacterial species in livestock farming is associated with foodborne human infections resulting in gastrointestinal and

post-infectious extra-intestinal morbidities with rising prevalence rates worldwide [2, 3]. Particularly *Campylobacter* infections are of substantial and increasing importance for food-borne diseases, whereas farm animals, especially poultry, are the main origin of human infection [4–6]. Following ingestion of raw or undercooked contaminated meat or surface water, symptoms of campylobacteriosis may vary considerably [7, 8]. Whereas some patients are even asymptomatic or present with rather mild symptoms, others suffer from abdominal cramps, fever, watery of even bloody and inflammatory diarrhea that usually resolve within 1 week. In rare cases, however, post-infectious sequelae such as Gullain-Barré syndrome, Miller Fisher syndrome, or reactive arthritis may manifest [9–11]. The pathogenesis of acute human campylobacteriosis is strongly triggered by the activation of innate immune responses via Toll-like Receptor-4 (TLR-4) mediated sensing of the bacterial lipooligosaccharide (LOS) that is expressed on the surface of *C. jejuni* [12, 13]. Thus, innate immune responses upon *C. jejuni* infection are very similar to those observed following peracute infections with other LOS expressing pathogens such as *Neisseria meningitidis* and *N. gonorrhoeae* [14, 15].

Terpenoids are antimicrobial compounds that are effective against a broad range of microorganisms [16]. Carvacrol (4-isopropyl-2-methylphenol) is a monoterpenoid which constitutes a major compound in essential oils of thyme and oregano and other medicinal plants with many proven health beneficial effects [17, 18]. Carvacrol modulates a multitude of different enzymatic functions which are causative for its anxiolytic, spasmolytic, cell regenerative and anticancer activities and is further in the focus of infection research due to its natural antimicrobial effects against several food-borne pathogens including *Campylobacter*. In vitro studies revealed that in bacteriostatic concentrations, carvacrol is capable of inducing changes in the fatty acid composition of the bacterial cell walls [19, 20]. In bactericidal concentrations, however, carvacrol even permeabilizes the outer membrane of Gram-negative bacteria [21]. In addition, carvacrol possesses ATPase-inhibiting activity [22, 23] and is proposed to act as a proton exchanger that reduces the pH gradient across the cytoplasmic membrane causing changes in proton motive force and in the ATP pool, which leads to cell death [23, 24]. Both, in vitro and in vivo studies revealed that carvacrol application could effectively reduce *C. jejuni* loads in intestinal samples derived from chicken [25–28]. Furthermore, carvacrol could effectively reduce virulence gene expression and invasion of *C. jejuni* into chicken cells [26, 29]. Most importantly, the finding that carvacrol application resulted in inhibition of motility and invasive properties of *C. jejuni* in vitro points towards carvacrol as a promising candidate molecule for the combat of human campylobacteriosis [30]. Recently, our group has established a clinical murine *C. jejuni* infection model allowing for pre-clinical studies of potential compounds against campylobacteriosis at the pharmaceutical level. After peroral *C. jejuni* infection, secondary abiotic IL-$10^{-/-}$ mice in which the intestinal microbiota had been depleted by antibiotic treatment could be stably colonized with *C. jejuni* at high pathogenic loads [12]. Given the lack of LOS resistance due to the absence of IL-10, these mice display *C. jejuni* induced acute enterocolitis within 1 week post-infection (p.i.) thereby mimicking clinical key features of severe campylobacteriosis [12, 31, 32]. In the present study, we applied this clinical murine model for human campylobacteriosis in order to investigate the therapeutic and/or even preventive efficacies of carvacrol treatment against *C. jejuni* colonization and immunopathological sequelae in vivo.

Results

Antimicrobial properties of carvacrol against *C. jejuni* isolates

We first addressed potential directed antimicrobial effects of carvacrol against *C. jejuni*. In vitro studies with 20 *C. jejuni* isolates including the reference strain 81–176 revealed carvacrol MIC_{90} values of 150 mg/l (pH 7.3).

Gastrointestinal pathogen loads following carvacrol treatment of *C. jejuni* infected mice

We next assessed potential health-beneficial properties (i.e., anti-*C. jejuni* and anti-inflammatory effects) of synthetic carvacrol (500 mg/l via the drinking water, ad libitum) in the here applied clinical murine infection model for the study of severe human campylobacteriosis. To accomplish this, secondary abiotic IL-$10^{-/-}$ mice were treated with carvacrol via the drinking water starting 4 days prior peroral *C. jejuni* infection with 10^9 bacterial cells by gavage on days 0 and 1. At day 6 p.i., placebo (PLC) treated control mice harbored median pathogen loads of 10^9 and 10^8 CFU/g in their colon and ileum, respectively, that were approximately 0.5 and 2.0 log orders of magnitude lower in mice from the carvacrol cohort, respectively ($p < 0.01$ and $p < 0.001$, respectively; Fig. 1). Hence, carvacrol treatment lowers intestinal *C. jejuni* burdens up to 2 orders of magnitude.

Clinical effects upon carvacrol treatment of *C. jejuni* infected mice

We further performed a daily survey of *C. jejuni* induced symptoms in infected mice applying a standardized clinical scoring system assessing gross appearance of mice, stool consistency and abundance of blood in fecal

samples. As early as 48 h after the latest of two peroral pathogenic challenges (i.e., on day 3 p.i.), mice from the carvacrol group displayed less severe *C. jejuni* induced disease as indicated by lower clinical scores as compared to PLC controls ($p < 0.001$; Additional file 1: Fig. S1), which also held true for days 5 and 6 p.i. ($p < 0.001$; Additional file 1: Fig. S1). At necropsy, PLC treated control mice suffered from acute enterocolitis characterized by wasting and bloody diarrhea (Fig. 2), whereas carvacrol treated mice, however, were clinically less compromised as indicated by significantly reduced scores for gross appearance, abundance of fecal blood and diarrhea ($p < 0.001$ vs. PLC; Fig. 2). Notably, all control mice, but only 10.5% of carvacrol treated animals presented with diarrhea at day 6 p.i. ($p < 0.001$; Fig. 2c). Hence, carvacrol treatment alleviates *C. jejuni* induced symptoms of campylobacteriosis in the here applied clinical murine infection model.

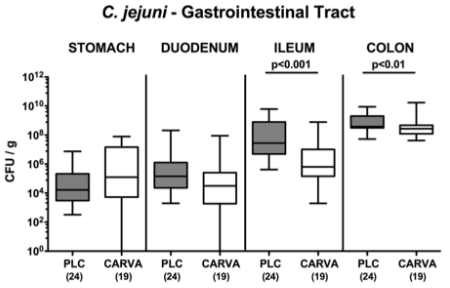


Fig. 1 Gastrointestinal pathogen loads following carvacrol treatment of *C. jejuni* infected mice. Starting 4 days prior peroral *C. jejuni* infection on days 0 and 1, secondary abiotic IL-$10^{-/-}$ mice were treated with synthetic carvacrol (CARVA; white boxes) or placebo (PLC; grey boxes) via the drinking water. At day 6 post-infection, *C. jejuni* were cultured from distinct luminal parts of the gastrointestinal tract and pathogen loads expressed as colony forming units per gram (CFU/g). Box plots represent the 75th and 25th percentiles of medians (black bar inside the boxes). The total range, significance levels (*p*-values) determined by the Mann–Whitney U test and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments

Apoptotic and regenerative responses in colonic epithelial cells upon carvacrol treatment of *C. jejuni* infected mice

We next assessed whether the beneficial effects of carvacrol treatment on the macroscopic outcome of *C. jejuni* infected mice could also be observed on microscopic level. To address this, we stained colonic paraffin sections with defined antibodies against distinct cell inflammatory and proliferative/regenerative markers as well as against distinct immune cell populations applying in situ immunohistochemistry. At day 6 p.i., mice of either cohort exhibited increased numbers of caspase3 positive apoptotic cells in their colonic epithelia ($p < 0.001$ vs.

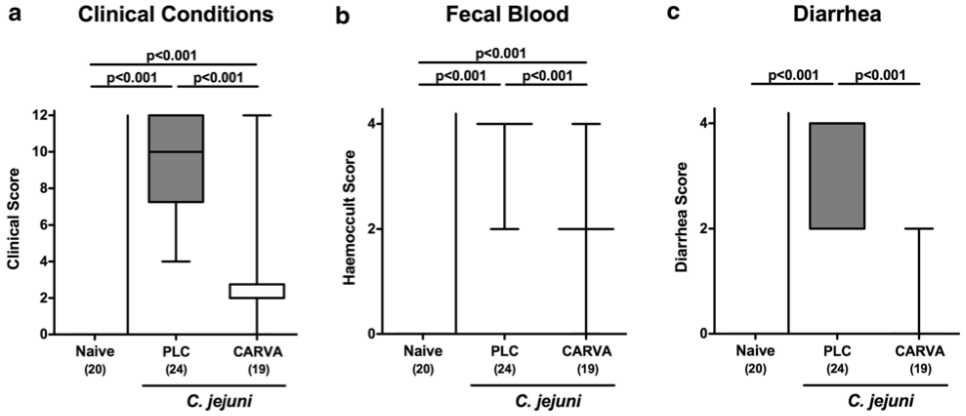


Fig. 2 Clinical outcome following carvacrol treatment of *C. jejuni* infected mice. Starting 4 days prior peroral *C. jejuni* infection on days 0 and 1, secondary abiotic IL-$10^{-/-}$ mice were treated with synthetic carvacrol (CARVA; white boxes) or placebo (PLC; grey boxes) via the drinking water. Applying a standardized clinical scoring system (see "Methods"), **a** clinical outcome, **b** abundance of fecal blood and **c** stool consistency were quantitated at day 6 post-infection. Box plots represent the 75th and 25th percentiles of medians (black bar inside the boxes). The total range, significance levels (*p*-values) determined by the Mann–Whitney U test and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments

naive; Fig. 3a). However, numbers of apoptotic cells were five times lower in colonic epithelia of carvacrol as compared to PLC treated mice at day 6 p.i. ($p < 0.001$; Fig. 3a; Additional file 2: Fig. S2A). We next stained large intestinal paraffin sections with antibodies against Ki67 and quantified the respective cell proliferation and regeneration counteracting *C. jejuni* induced cell damage microscopically. In fact, *C. jejuni* infection was accompanied by a marked increase in Ki67 positive colonic epithelial cells ($p < 0.001$ vs. naive; Fig. 3b; Additional file 2: Fig. S2B). Importantly, numbers of proliferative/ regenerative cells were significantly elevated in carvacrol as compared to PLC mice at day 6 p.i. ($p < 0.001$; Fig. 3b; Additional file 2: Fig. S2B). Hence, carvacrol treatment ameliorates murine campylobacteriosis by inhibiting apoptosis and stimulating regenerative processes in the colonic epithelia.

Colonic immune responses upon carvacrol treatment of *C. jejuni* infected mice

We next quantitatively assessed large intestinal immune responses upon carvacrol treatment of *C. jejuni* infected mice. Within 6 days following *C. jejuni* infection, distinct immune cell populations such as CD3+ T lymphocytes cells and B220+ B lymphocytes had multifold increased in the colonic mucosa and lamina propria of mice from either cohort ($p < 0.001$; Fig. 3c, d; Additional file 2: Fig. S2C, D). Notably, colonic T lymphocytes, however, were lower following carvacrol as compared to PLC treatment at day 6 p.i. ($p < 0.005$, Fig. 3c; Additional file 2: Fig. S2C). The increased large intestinal abundances of immune cells upon *C. jejuni* infection were accompanied by enhanced secretion of pro-inflammatory mediators such as nitric oxide (NO), tumor necrosis factor (TNF) and interleukin (IL)-6 in colonic ex vivo biopsies obtained at day 6 p.i. ($p < 0.05$–0.001; Fig. 4a, c, d). Carvacrol treatment of *C. jejuni* infected mice, however, resulted in lower colonic NO, interferon (IFN)-γ and TNF concentrations as compared to PLC application ($p < 0.05$–0.001; Fig. 4a–c). Of note, IFN-γ levels measured in the large intestines of carvacrol treated mice at day 6 p.i. did not differ from those of naive control mice (n.s.; Fig. 4b). Hence, carvacrol dampened *C. jejuni* induced colonic inflammation.

Ileal inflammatory immune responses upon carvacrol treatment of *C. jejuni* infected mice

In humans and murine infection models, *C. jejuni* induced intestinal inflammation is considered to primarily affect the large intestinal tract resulting in acute enterocolitis [9]. Nevertheless, we expanded our intestinal inflammatory survey of campylobacteriosis induced in secondary abiotic IL-$10^{-/-}$ mice to the small intestines. At day 6 p.i., PLC, but not carvacrol treated mice displayed almost three time higher numbers of apoptotic ileal epithelial cells ($p < 0.001$ vs. naive; Additional file 3: Fig. S3A and Additional file 4: Fig. S4A), which was accompanied by increased B cell counts in the mucosa and lamina propria of *C. jejuni* infected mice from the PLC, but not carvacrol cohort ($p < 0.001$; Additional file 3: Fig. S3D and Additional file 4: Fig. S4D). In addition, irrespective of the treatment regimen, mice displayed increased numbers of proliferating ileal epithelial cells ($p < 0.05$–0.001; Additional file 3: Fig. S3B and Additional file 4: Fig. S4B) as well as of T lymphocytes ($p < 0.001$; Additional file 3: Fig. S3C and Additional file 4: Fig. S4C) in the small intestinal mucosa and lamina propria. The inflammation-ameliorating effects of carvacrol treatment also in the small intestinal tract of *C. jejuni* infected mice was further supported by increased secretion of pro-inflammatory cytokines such as TNF and IFN-γ in ileal ex vivo biopsies taken from PLC ($p < 0.05$–0.005 vs. naive), but not carvacrol treated mice at day 6 p.i. ($p < 0.05$ vs. PLC; Additional file 5: Fig. S5). Hence, the campylobacteriosis ameliorating properties of carvacrol were not restricted to the large intestinal tract, but also effective in the distal small intestines.

Inflammatory immune responses in MLN upon carvacrol treatment of *C. jejuni* infected mice

We next surveyed disease ameliorating effects of carvacrol treatment in the MLN of *C. jejuni* infected mice. In support of our results obtained from the colon and ileum, less distinct secretion of pro-inflammatory mediators such as NO, IFN-γ, TNF and IL-6 could be assessed in MLN of carvacrol as compared to PLC treated mice at day 6 p.i. ($p < 0.01$–0.001; Fig. 5). Of note, the concentrations of the three latter cytokines measured in carvacrol treated *C. jejuni* infected mice did not differ from those obtained from naive control animals (n.s.; Fig. 5b–d). Hence, the anti-inflammatory properties of carvacrol in *C. jejuni* infected mice were also effective in MLN draining the intestinal tract.

Extra-intestinal including systemic inflammatory immune responses upon carvacrol treatment of *C. jejuni* infected mice

We further assessed disease-alleviating properties of carvacrol treatment in extra-intestinal including systemic compartments of *C. jejuni* infected mice. Increased numbers of caspase3 positive apoptotic cells could be observed in paraffin sections taken from liver, kidneys and lungs at day 6 p.i. ($p < 0.005$–0.001), whereas apoptotic cell counts in either organs were lower in carvacrol as compared to PLC treated mice ($p < 0.05$–0.001; Fig. 6; Additional file 6: Fig. S6). Of

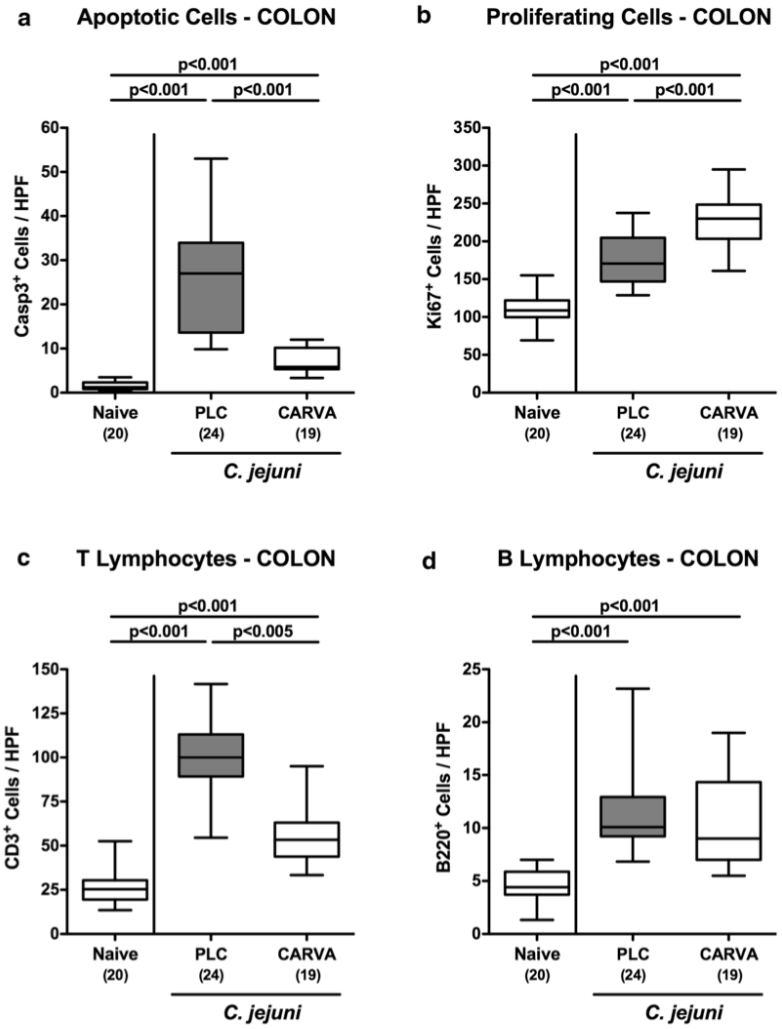


Fig. 3 Large intestinal apoptotic, proliferative/regenerative and immune cell responses upon carvacrol treatment of *C. jejuni* infected mice. Starting 4 days prior peroral *C. jejuni* infection on days 0 and 1, secondary abiotic IL-10$^{-/-}$ mice were treated with synthetic carvacrol (CARVA; white boxes) or placebo (PLC; grey boxes) via the drinking water. The average numbers of colonic epithelial **a** apoptotic cells (positive for caspase-3, Casp3) and **b** proliferating/regenerating cells (positive for Ki67) as well as of **c** T lymphocytes (positive for CD3) and **d** B lymphocytes (positive for B220) in the mucosa of lamina propria from six high power fields (HPF, 400x magnification) per mouse were assessed microscopically in immunohistochemically stained large intestinal paraffin sections at day 6 post-infection. Naive mice served as uninfected controls. The total range, significance levels (p-values) determined by the Mann–Whitney U test and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments

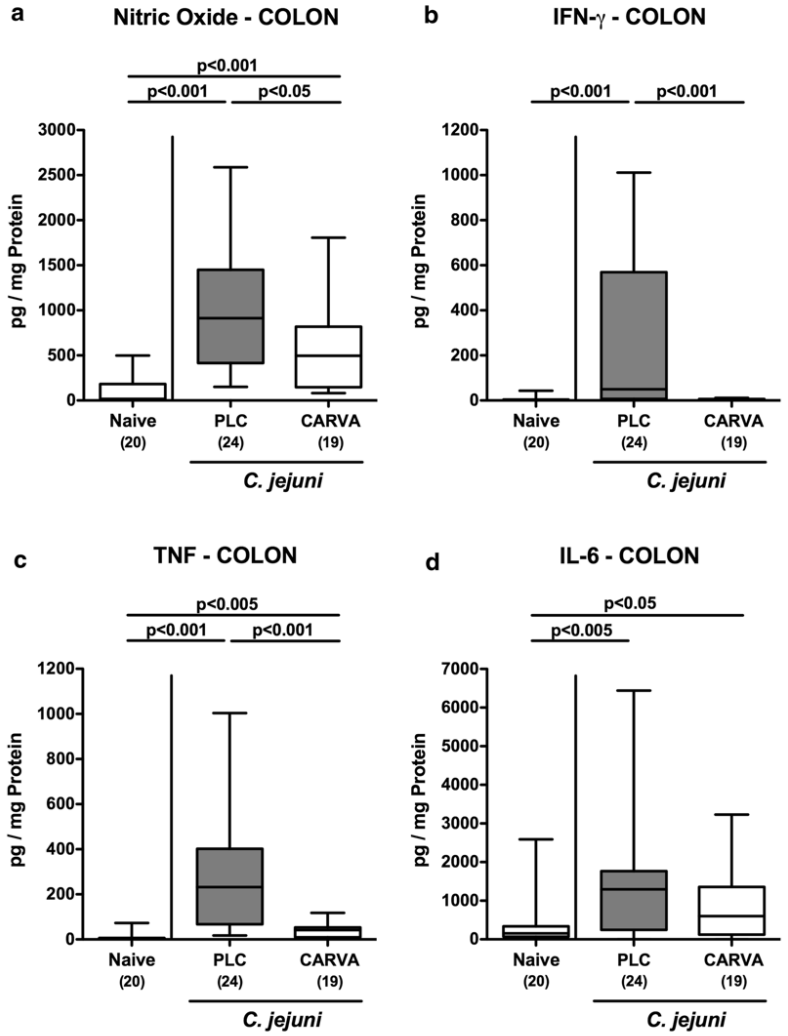


Fig. 4 Colonic pro-inflammatory mediator secretion following carvacrol treatment of *C. jejuni* infected mice. Starting 4 days prior peroral *C. jejuni* infection on days 0 and 1, secondary abiotic IL-10$^{-/-}$ mice were treated with synthetic carvacrol (CARVA; white boxes) or placebo (PLC; grey boxes) via the drinking water. **a** Nitric oxide, **b** IFN-γ, **c** TNF, and **d** IL-6 concentrations were measured in supernatants of colonic ex vivo biopsies derived at day 6 post-infection. Naive mice served as uninfected controls. The total range, significance levels (p-values) determined by the Mann–Whitney U test and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments

note, in lungs numbers of apoptotic cells did not differ in carvacrol treated *C. jejuni* infected and naive mice (n.s.; Fig. 6c, Additional file 6: Fig. S6C).

We next measured pro-inflammatory cytokine secretion in respective ex vivo biopsies. At day 6 p.i., lower IFN-γ as well as TNF concentrations could be assessed

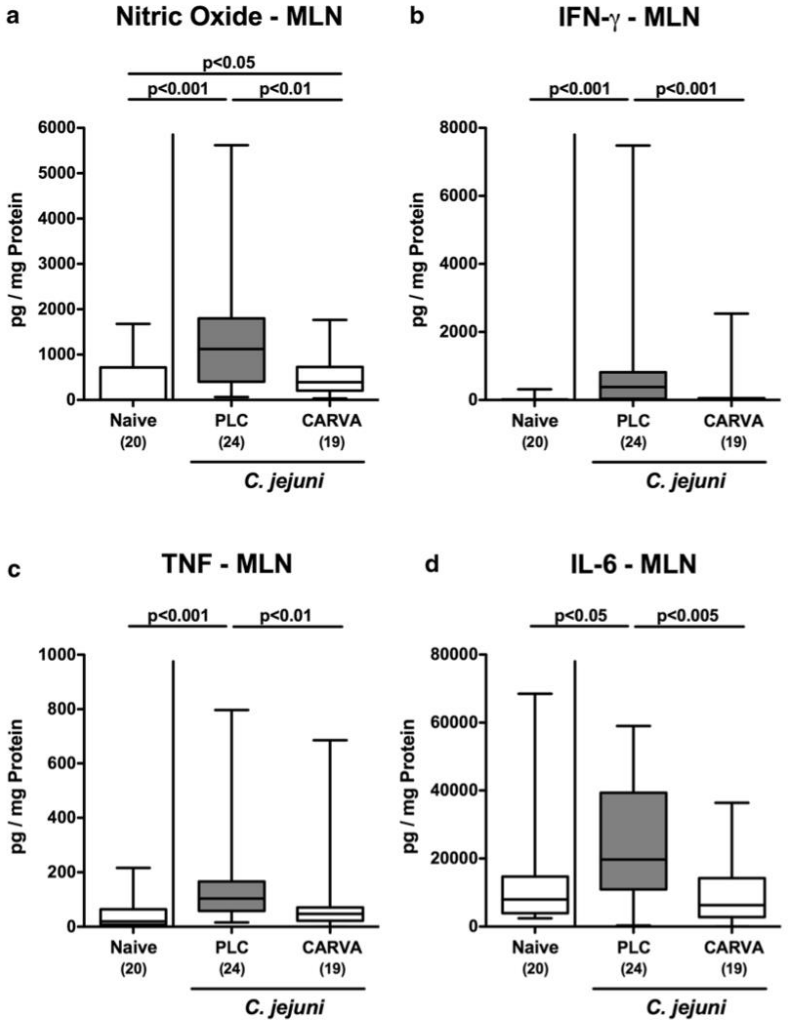


Fig. 5 Pro-inflammatory mediator secretion in mesenteric lymph nodes following carvacrol treatment of *C. jejuni* infected mice. Starting 4 days prior peroral *C. jejuni* infection on days 0 and 1, secondary abiotic IL-10$^{-/-}$ mice were treated with synthetic carvacrol (CARVA; white boxes) or placebo (PLC; grey boxes) via the drinking water. **a** Nitric oxide, **b** IFN-γ, **c** TNF, and **d** IL-6 concentrations were measured in supernatants of ex vivo biopsies derived from mesenteric lymph nodes (MLN) at day 6 post-infection. Naive mice served as uninfected controls. The total range, significance levels (p-values) determined by the Mann–Whitney U test and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments

in the liver of carvacrol as compared to PLC treated mice ($p < 0.05$ and $p < 0.001$, respectively; Fig. 7a, b), which also held true for renal IFN-γ protein levels ($p < 0.05$; Fig. 7c), but not TNF concentration (n.s.; Fig. 7d). Moreover, IFN-γ concentrations increased upon *C. jejuni* infection in the lungs of PLC ($p < 0.05$; Fig. 7e), but not carvacrol treated mice (n.s.; Fig. 7e), whereas elevated pulmonary TNF levels could be obtained at day 6 p.i.,

irrespective of the treatment regimen ($p < 0.05$; Fig. 7f). Remarkably, the inflammation-dampening effects of carvacrol in *C. jejuni* infected mice could also be observed systemically as indicated by lower increases in pro-inflammatory mediators such as IFN-γ, TNF, monocyte chemoattractant protein-1 (MCP-1) and IL-6 measured in serum samples taken from carvacrol as compared to PLC treated mice at day 6 p.i. ($p < 0.05$–0.005; Fig. 8). Hence, carvacrol treatment dampens *C. jejuni* induced pro-inflammatory responses also in extra-intestinal and even systemic compartments.

Bacterial translocation into extra-intestinal including systemic compartments of carvacrol treated mice following *C. jejuni* infection

We finally addressed whether carvacrol treatment had an impact on the translocation of viable pathogens originating from the gastrointestinal tract to extra-intestinal including systemic tissue sites. Whereas *C. jejuni* could be cultured from MLN of PLC and carvacrol treated mice in 45.8 and 42.1% of cases at day 6 p.i., respectively (Fig. 9a), pathogenic translocation rates were lower in spleens (15.8% vs. 20.8%), livers (5.3% vs. 8.3%), kidneys (0% vs. 8.3%) and lungs (0% vs 12.5%) taken from mice of the carvacrol versus PLC cohorts (Fig. 9b–e). Of note, all blood cultures were *C. jejuni* negative (Fig. 9f). Hence, carvacrol treatment of *C. jejuni* infected mice is accompanied with less frequent translocation of viable pathogens from the intestinal tract to extra-intestinal compartments.

Discussion

In the European Union the annual financial burden to the public health systems and to loss of individual health and productivity due to foodborne *C. jejuni* infections are estimated to account for 2.4 billion Euro [33]. Given the progressive emergence of human campylobacteriosis, identification of non-synthetic antibiotic molecules that

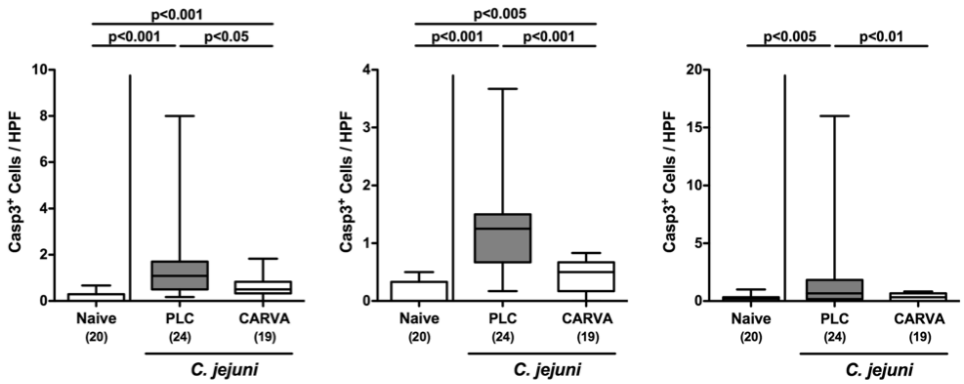
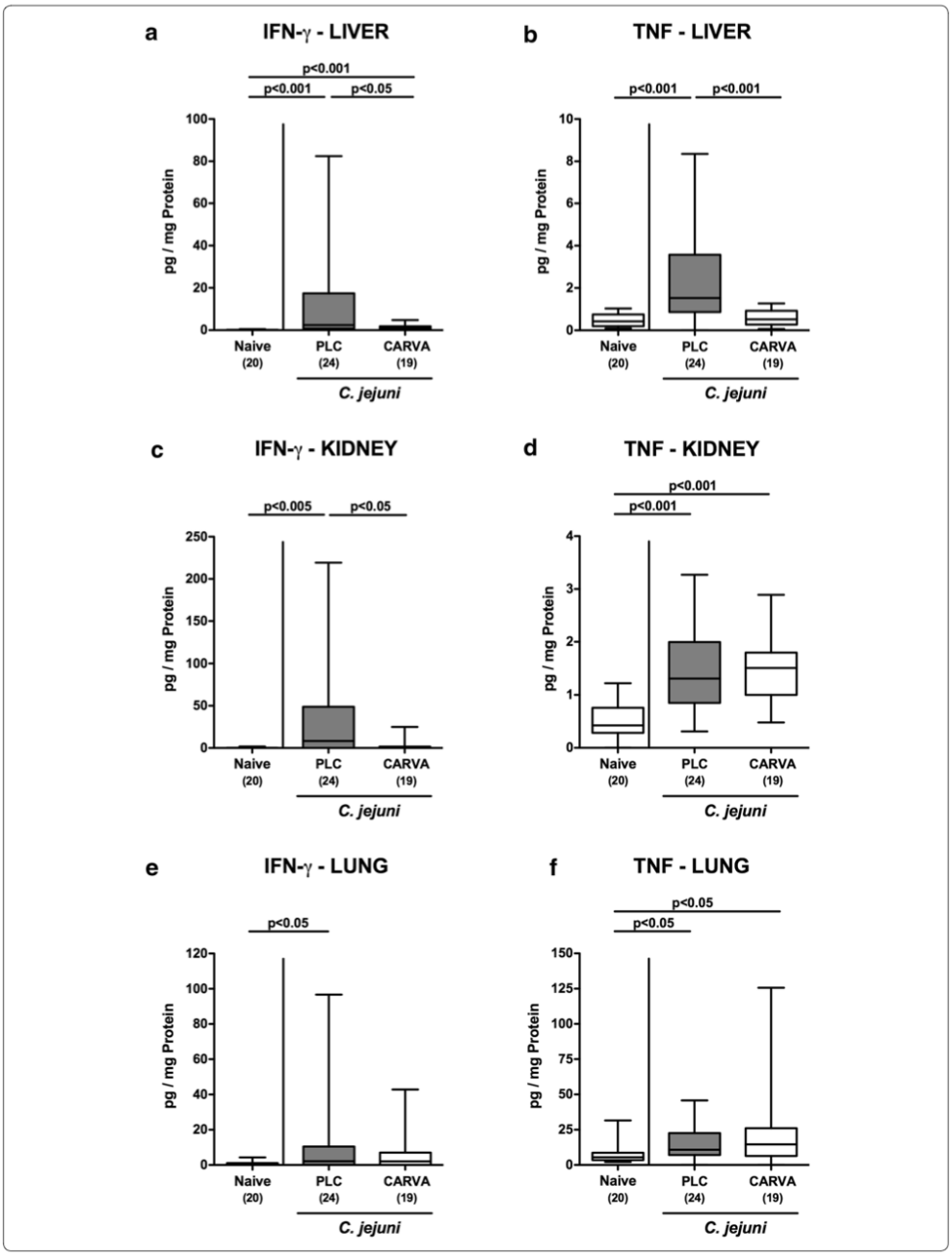


Fig. 6 Extra-intestinal apoptosis following carvacrol treatment of *C. jejuni* infected mice. Starting 4 days prior peroral *C. jejuni* infection on days 0 and 1, secondary abiotic IL-$10^{-/-}$ mice were treated with synthetic carvacrol (CARVA; white boxes) or placebo (PLC; grey boxes) via the drinking water. The average numbers of apoptotic cells (positive for caspase-3, Casp3) from six high power fields (HPF, ×400 magnification) per mouse were assessed microscopically in immunohistochemically stained paraffin sections derived from **a** liver, **b** kidneys and **c** lungs at day 6 post-infection. Naive mice served as uninfected controls. The total range, significance levels (p-values) determined by the Mann–Whitney U test and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments

(See figure on next page.)

Fig. 7 Extra-intestinal pro-inflammatory mediator secretion following carvacrol treatment of *C. jejuni* infected mice. Starting 4 days prior peroral *C. jejuni* infection on days 0 and 1, secondary abiotic IL-$10^{-/-}$ mice were treated with synthetic carvacrol (CARVA; white boxes) or placebo (PLC; grey boxes) via the drinking water. **a**, **c**, **e** IFN-γ and **b**, **d**, **f** TNF concentrations were measured in supernatants of ex vivo biopsies derived from **a**, **b** liver, **c**, **d** kidneys and **e**, **f** lungs at day 6 post-infection. Naive mice served as uninfected controls. The total range, significance levels (p-values) determined by the Mann–Whitney U test and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments


a
IFN-γ - LIVER
p<0.001
p<0.001
p<0.05
pg / mg Protein
Naive (20)
PLC (24)
CARVA (19)
C. jejuni
b
TNF - LIVER
p<0.001
p<0.001
c
IFN-γ - KIDNEY
p<0.005
p<0.05
d
TNF - KIDNEY
p<0.001
p<0.001
e
IFN-γ - LUNG
p<0.05
f
TNF - LUNG
p<0.05
p<0.05

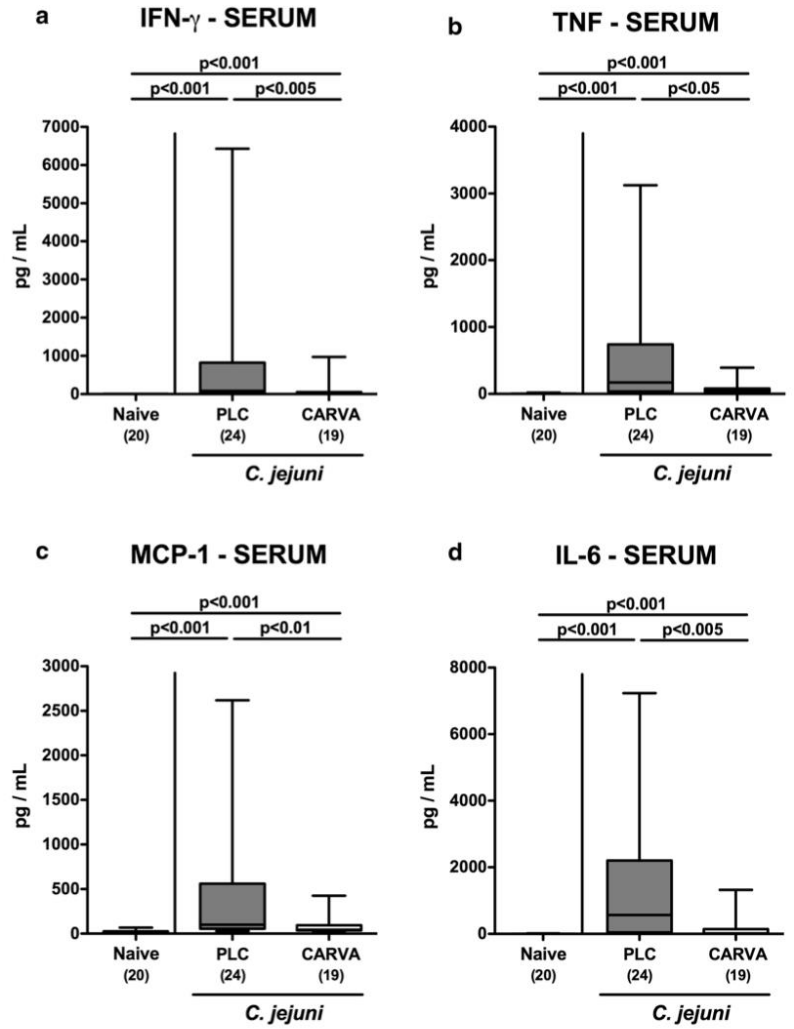


Fig. 8 Systemic pro-inflammatory mediator secretion following carvacrol treatment of *C. jejuni* infected mice. Starting 4 days prior peroral *C. jejuni* infection on days 0 and 1, secondary abiotic IL-10$^{-/-}$ mice were treated with synthetic carvacrol (CARVA; white boxes) or placebo (PLC; grey boxes) via the drinking water. **a** IFN-γ, **b** TNF, **c** MCP-1 and **d** IL-6 concentrations were measured serum samples taken at day 6 post-infection. Naive mice served as uninfected controls. The total range, significance levels (p-values) determined by the Mann–Whitney U test and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments

exert both, potent anti-*Campylobacter* and anti-inflammatory effects in infected individuals is highly appreciable in order to combat pathogen-induced morbidities. The phenolic compound carvacrol has been shown to exert antimicrobial activities by increasing the membrane permeability of Gram-negative bacteria including

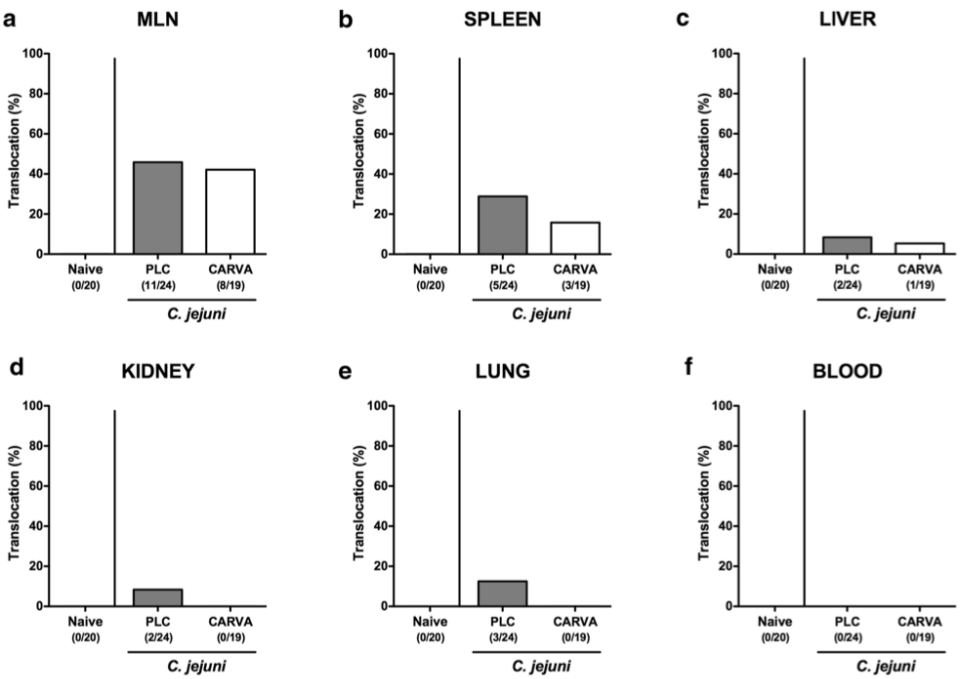


Fig. 9 Bacterial translocation following carvacrol treatment of *C. jejuni* infected mice. Starting 4 days prior peroral *C. jejuni* infection on days 0 and 1, secondary abiotic IL-10$^{-/-}$ mice were treated with synthetic carvacrol (CARVA; white boxes) or placebo (PLC; grey boxes) via the drinking water. At day 6 post-infection, the abundance of *C. jejuni* was assessed in ex vivo biopsies derived from **a** MLN, **b** spleen, **c** liver, **d** kidneys, **e** lungs and **f** cardiac blood by culture. The cumulative relative translocation rate of viable pathogens into the respective compartment out of four independent experiments is indicated (in %)

C. jejuni [34] and thus, represents a promising option to replace conventionally used synthetic antibiotics for lowering the *C. jejuni* burden in livestock animals and avoiding the development of antibiotic resistant *C. jejuni* strains. In fact, the European Union has approved carvacrol as animal food supplement on the basis of results derived from carvacrol treatment studies in chicken farms in order to reduce the abundance of *Campylobacter* in poultry farming and meat production [35]. Notably, carvacrol constitutes a major component of oregano and thyme oils representing a "generally as safe recognized" phytoherbal compound [36–40] that has been pharmaceutically approved for the treatment of gastrointestinal morbidities in humans [41, 42]. However, scientifically validated data regarding carvacrol application against *C. jejuni* induced enterocolitis in humans are scarce.

In the present pre-clinical intervention study we therefore applied a well-established clinical *C. jejuni* infection model of acute campylobacteriosis by using secondary abiotic IL-10$^{-/-}$ mice which display the clinical hallmarks of severe campylobacteriosis seen in human patients. In our study, we applied carvacrol via the drinking water in a concentration of 500 mg/l that was more than three times higher than the MIC_{90} value of 150 mg/l that had been determined in 20 *C. jejuni* insolates before. Carvacrol treatment starting 4 days prior murine infection could sufficiently lower intestinal *C. jejuni* burdens up to 2 orders of magnitude until day 6 p.i. The antimicrobial properties of carvacrol directed against food-borne pathogens such as *C. jejuni* [26, 29, 30, 37] *Salmonella* spp. [37, 43–45], *Escherichia coli* O157:H7 [46] and *Bacillus cereus* [47, 48] have been shown in vitro recently. Synthetic carvacrol was, however, more effective against Gram-negative bacteria than against Gram-positive species [49].

Remarkably, carvacrol treatment alleviated *C. jejuni* induced symptoms of campylobacteriosis including wasting and bloody diarrhea. Previous in vitro studies revealed that carvacrol reduced *C. jejuni* virulence by inhibition of motility, cell attachment, tissue invasion and toxin production and disrupted bacterial ATP production leading to bacterial cell death [22–27, 29, 30, 50]. In addition, carvacrol reduced *Campylobacter* colonization in chicken in vivo and inhibited biofilm formation [26]. Ameliorated murine campylobacteriosis was further characterized by less distinct *C. jejuni* induced apoptosis in both, colonic and ileal epithelia, whereas intestinal cell proliferative/regenerative properties counteracting pathogen-induced cell damage were enhanced upon carvacrol treatment. Furthermore, carvacrol application dampened intestinal pro-inflammatory immune responses upon *C. jejuni* infection as indicated by less abundance of T and B lymphocytes in the mucosa and lamina propria of both, the large and the small intestines and lower concentrations of pro-inflammatory mediators including TNF and IL-6 in the intestinal tract and further, in the MLN draining the infected intestines. These results are supported by recent in vitro studies showing that carvacrol treatment of both, stimulated dendritic cells and macrophages resulted in less distinct TNF and IFN-γ secretion [51], whereas carvacrol could down-regulate lipopolysaccharide (LPS) induced expression of pro-inflammatory cytokines such as TNF and IL-6 in broilers [52].

Notably, the inflammation-dampening properties of exogenous carvacrol was not restricted to the intestinal tract, but could also be observed in extra-intestinal organs such as liver, kidneys and lungs as indicated by less distinct apoptosis and secretion of the pro-inflammatory cytokines TNF and IFN-γ in respective organs. In support, carvacrol has been shown to improve survival during LPS-induced endotoxinemia and acute lung injury in mice and to result in less distinct secretion of pro-inflammatory cytokines including TNF and IL-6 [53].

Strikingly, the potent campylobacteriosis ameliorating effects upon carvacrol treatment could also be assessed systemically given that serum concentrations of TNF, IFN-γ, MCP-1 and IL-6 were lower in carvacrol as compared to PLC treated *C. jejuni* infected mice. Of note, translocation of viable pathogens from the intestinal tract to extra-intestinal organs occurred less frequently in the carvacrol versus PLC cohort. Even though all blood cultures were *C. jejuni* negative, one needs to take further into account that soluble bacterial molecules such as LOS and other cell wall constituents might have been transported via the circulation and been responsible for the deleterious outcome of severe campylobacteriosis in PLC control mice.

Overall, it is tempting to speculate that the observed disease-ameliorating properties of carvacrol are due to an orchestrated sum effect of distinct anti-inflammatory features of the compound: (i) lower intestinal pathogen loads and hence, (ii) less LOS exposure less distinctly inducing pro-inflammatory mediator secretion, (iii) hypothetical direct anti-LOS effect of carvacrol, (iv) interfering with distinct *C. jejuni* virulence factors by blocking motility, adhesion, invasion, LOS expression, leading to (v) less recruitment of immune cells resulting in (vi) less secretion of pro-inflammatory mediators, (vii) less cytotoxicity including apoptosis, (viii) more counter-regulatory cell proliferation/regeneration, (ix) less pathogenic translocation, and (x) less extra-intestinal including (xi) systemic immune responses.

Taken together these beneficial effects result in a significant amelioration of disease and better overall clinical outcome of mice in the clinical infection model for human campylobacteriosis.

Conclusion

The lowered *C. jejuni* loads and alleviated symptoms observed in the here applied clinical murine model for human campylobacteriosis highlight the application of carvacrol as a promising option not only for the treatment of campylobacteriosis in humans and hence, for prevention of post-infectious sequelae, but also for the reduction of *C. jejuni* colonization in lifestock animals.

Methods

Determination of minimal inhibitory concentrations of carvacrol

For determination of minimal inhibitory concentration (MIC) values of carvacrol, 20 *C. jejuni* isolates including the reference strain 81–176 used for infection of mice (see below) were tested for their antimicrobial susceptibility applying the broth microdilution method. Procedures regarding inoculum density, growth medium, incubation time and conditions were performed in accordance with the recommendations given in the Clinical and Laboratory Standards Institute (CLSI) document VET01-Ed5. Twofold serial dilutions ranging from 0.008 to 8.0 mmol/l (1–1202 µg/ml) for carvacrol were tested. Stock solutions were prepared in Mueller–Hinton broth and adjusted to pH 7.3. The *C. jejuni* reference strain DSM 4688 was used for quality control purposes. The MIC value of the reference strain was tested in advance in three independent experiments using the broth microdilution method and the broth macrodilution method.

Generation of secondary abiotic mice

IL-10$^{-/-}$ mice (female and male, all in C57BL/6j background) were reared under specific pathogen free (SPF) conditions in the same unit of the Forschungseinrichtungen für Experimentelle Medizin (FEM,

Charité—University Medicine Berlin). In order to counteract physiological colonization resistance and thus assure stable gastrointestinal *C. jejuni* colonization [13], secondary abiotic mice with a depleted gut microbiota were generated as described earlier [13, 54]. In brief, immediately post weaning 3-week-old mice were subjected to a 10-week course of broad-spectrum antibiotic treatment by adding ampicillin plus sulbactam (1 g/l; Ratiopharm, Germany), vancomycin (500 mg/l; Cell Pharm, Germany), ciprofloxacin (200 mg/l; Bayer Vital, Germany), imipenem (250 mg/l; MSD, Germany) and metronidazole (1 g/l; Fresenius, Germany) to the autoclaved drinking water (ad libitum). Two days before pathogenic challenge the antibiotic cocktail was replaced by autoclaved tap water to assure antibiotic washout.

Carvacrol treatment

Four days prior *C. jejuni* infection treatment with carvacrol (Sigma-Aldrich, Munich, Germany; daily dose of 100 mg carvacrol per kg body weight) was initiated by dissolving the compound in Tween 80 (0.2% v/v) to a final concentration of 500 mg/l autoclaved tap water (ad libitum). Placebo control mice received Tween 80 only.

Campylobacter jejuni infection

Twelve-week old mice were perorally challenged with 10^9 colony forming units (CFU) of the highly pathogenic *C. jejuni* reference strain 81–176 by gavage (in a total volume of 0.3 ml phosphate buffered saline (PBS), Gibco, Life Technologies, UK). Animals were continuously maintained in a sterile environment (autoclaved food and drinking water or sterile antibiotic cocktail) and handled under strict aseptic conditions in order to avoid contaminations.

Clinical conditions

Before and after *Campylobacter jejuni* infection clinical conditions of mice were assessed on a daily basis applying a standardized cumulative clinical score (maximum 12 points) addressing the abundance of blood in feces (0: no blood; 2: microscopic detection of blood by the Guajac method using Haemoccult, Beckman Coulter/PCD, Germany; 4: macroscopic blood visible), diarrhea (0: formed feces; 2: pasty feces; 4: liquid feces), and the clinical aspect (0: normal; 2: ruffled fur, less locomotion; 4: isolation, severely compromised locomotion, pre-final aspect) as described earlier [55].

Sampling procedures

At day 6 p.i., mice were sacrificed by isofluran inhalation (Abbott, Germany). Luminal gastrointestinal samples (i.e., from stomach, duodenum, ileum and colon) and ex vivo biopsies were taken from colon, mesenteric lymph nodes (MLN), liver, kidneys, lungs, and spleen under sterile conditions. Intestinal samples were collected from each mouse in parallel for microbiological, immunohistopathological and immunological analyses. The absolute colonic lengths were measured with a ruler (in cm).

Pathogenic colonization and translocation

Campylobacter jejuni loads were surveyed in fecal samples over time p.i., and upon necropsy in luminal samples taken from the stomach, duodenum, ileum and colon as well as in homogenates of ex vivo biopsies derived from MLN, spleen, liver, kidney and lung as well as in cardiac blood samples by culture as described previously [13, 56]. In brief, intraluminal gastrointestinal samples and respective ex vivo biopsies were homogenized in sterile PBS with a pistil and serial dilutions plated onto karmali agar (Oxoid, Wesel, Germany) and incubated in a microaerophilic atmosphere for at least 48 h. Cardiac blood (0.2 ml) was immediately streaked onto karmali agar plates. The detection limit of viable pathogens was approximately 100 CFU/g.

Immunohistochemistry

In situ immunohistochemical analyses were performed in ex vivo biopsies derived from colon, ileum, liver, kidneys, and lungs that had been immediately fixed in 5% formalin and embedded in paraffin as stated elsewhere [32, 57–59]. In brief, in order to detect apoptotic epithelial cells, proliferation epithelial cells, T lymphocytes, and B lymphocytes, paraffin sections (5 µm) were stained with primary antibodies directed against cleaved caspase 3 (Asp175, Cell Signaling, Beverly, MA, USA, 1:200), Ki67 (TEC3, Dako, Denmark, 1:100), CD3 (#N1580, Dako, 1:10), and B220 (No. 14–0452-81, eBioscience; 1:200), respectively. After incubation with the primary antibody (30 min), sections were incubated for another 30 min with the respective secondary antibody (for anti-cleaved caspase 3 and anti-CD3 staining: biotinylated donkey anti-rabbit antibody; for anti-Ki67 and anti-B220 staining: biotinylated rabbit anti-rat antibody; all purchased from Dianova, Hamburg, Germany). The Streptavidin–Alkaline Phosphatase Kit (Dako) using Fast Red as chromogen was applied as detection system. Negative controls were generated in samples in which the respective primary antibody had been omitted. Positively stained cells were then examined by light microscopy (magnification 100× and 400×), and for each mouse the average number of respective positively stained cells was determined within at least six high power fields (HPF, 0.287 mm^2, 400× magnification) by a blinded independent investigator.

Pro-inflammatory mediators

Colonic and ileal ex vivo biopsies were cut longitudinally and washed in PBS. Ex vivo biopsies derived from liver (approximately 1 cm^3), kidney (one half after longitudinal cut), lung, MLN (3–4 lymph nodes) or strips of approximately 1 cm^2 colonic or ileal tissues were placed in 24-flat-bottom well culture plates (Nunc, Germany) containing 500 µl serum-free RPMI 1640 medium (Gibco, life technologies, UK) supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml; PAA Laboratories, Germany). After 18 h at 37 °C, culture supernatants and serum samples were tested for IFN-γ, TNF, IL-6, and MCP-1 by the Mouse Inflammation Cytometric Bead Assay (CBA; BD Biosciences, Germany) on a BD FACSCanto II flow cytometer (BD Biosciences). Systemic pro-inflammatory cytokine concentrations were measured in serum samples. NO concentrations were assessed by the Griess reaction [54, 60].

Statistical analysis

Medians and levels of significance were determined using Mann–Whitney test (GraphPad Prism v7, USA) as indicated. Two-sided probability (p) values ≤ 0.05 were considered significant. Experiments were reproduced three times.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13099-019-0343-4.

Additional files

Additional file 1: Figure S1. Kinetic survey of clinical conditions following carvacrol treatment of *C. jejuni* infected mice. Starting 4 days prior peroral *C. jejuni* infection on day (d) 0 and d1, secondary abiotic IL-$10^{-/-}$ mice were treated with synthetic carvacrol (CARVA; white boxes) or placebo (PLC; grey boxes) via the drinking water. Severities of clinical symptoms were surveyed daily from d0 until d6 applying a standardized clinical scoring system postinfection (see "Methods"). Box plots represent the 75th and 25th percentiles of medians (black bar inside the boxes). Total range, significance levels (p-values; determined between groups at respective time points; ***$p < 0.001$) by the Mann–Whitney U test and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments.

Additional file 2: Figure S2. Representative photomicrographs illustrating apoptotic and proliferating epithelial as well as immune cells responses in large intestines upon carvacrol treatment of *C. jejuni* infected mice. Starting 4 days prior peroral *C. jejuni* infection on days 0 and 1, secondary abiotic IL-$10^{-/-}$ mice were treated with synthetic carvacrol (CARVA) or placebo (PLC) via the drinking water. Naive mice served as uninfected controls. Photomicrographs reepresentative for four independent experiments illustrate the average numbers of (**A**) apoptotic epithelial cells (Casp3+), (**B**) proliferating epithelial cells (Ki67+), (**C**) T lymphocytes (CD3+), and (**D**) B lymphocytes (B220+) in at least six high power fields (HPF) as quantitatively assessed in colonic paraffin sections applying in situ immunohistochemistry at day 6 post-infection (**A**: 400× magnification, scale bar 20 µm; **B**–**D**: 100× magnification, scale bar 100 µm).

Additional file 3: Figure S3. Ileal apoptotic, proliferative and immune cell responses upon carvacrol treatment of *C. jejuni* infected mice. Starting 4 days prior peroral *C. jejuni* infection on days 0 and 1, secondary abiotic IL-$10^{-/-}$ mice were treated with synthetic carvacrol (CARVA; white boxes) or placebo (PLC; grey boxes) via the drinking water. The average numbers of (**A**) apoptotic (positive for caspase3, Casp3) and (**B**) proliferative/regenerative (positive for Ki67) ileal epithelial cells as well as of (**C**) T lymphocytes (positive for CD3) and (**D**) B lymphocytes (positive for B220) in the ileal mucosa and lamina propria from six high power fields (HPF, 400× magnification) per mouse were assessed microscopically in immunohistochemically stained small intestinal paraffin sections at day 6 post-infection. Naive mice served as uninfected controls. The total range, significance levels (p-values) determined by the Mann–Whitney U test and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments.

Additional file 4: Figure S4. Representative photomicrographs illustrating apoptotic and proliferating epithelial as well as immune cells responses in large intestines upon carvacrol treatment of *C. jejuni* infected mice. Starting 4 days prior peroral *C. jejuni* infection on days 0 and 1, secondary abiotic IL-$10^{-/-}$ mice were treated with synthetic carvacrol (CARVA) or placebo (PLC) via the drinking water. Naive mice served as uninfected controls. Photomicrographs reepresentative for four independent experiments illustrate the average numbers of (**A**) apoptotic epithelial cells (Casp3+), (**B**) proliferating epithelial cells (Ki67+), (**C**) T lymphocytes (CD3+), and (**D**) B lymphocytes (B220+) in at least six high power fields (HPF) as quantitatively assessed in ileal paraffin sections applying in situ immunohistochemistry at day 6 post-infection (**A**: 400× magnification, scale bar 20 µm; **B**–**D**: 100× magnification, scale bar 100 µm).

Additional file 5: Figure S5. Ileal pro-inflammatory mediator secretion in carvacrol treated mice following *C. jejuni* infection. Starting 4 days prior peroral *C. jejuni* infection on days 0 and 1, secondary abiotic IL-$10^{-/-}$ mice were treated with synthetic carvacrol (CARVA; white boxes) or placebo (PLC; grey boxes) via the drinking water. (**A**) IFN-γ and (**B**) TNF concentrations were measured in supernatants of ileal ex vivo biopsies derived at day 6 post-infection. Naive mice served as uninfected controls. The total range, significance levels (p-values) determined by the Mann–Whitney U test and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments.

Additional file 6: Figure S6. Representative photomicrographs illustrating apoptotic cells responses in extra-intestinal compartments upon carvacrol treatment of *C. jejuni* infected mice. Starting 4 days prior peroral *C. jejuni* infection on days 0 and 1, secondary abiotic IL-$10^{-/-}$ mice were treated with synthetic carvacrol (CARVA) or placebo (PLC) via the drinking water. Naive mice served as uninfected controls. Photomicrographs reepresentative for four independent experiments illustrate the average numbers of apoptotic cells (Casp3+) in (**A**) liver, (**B**) kidney and (**C**) lung in at least six high power fields (HPF) as quantitatively assessed in paraffin sections of respective ex vivo biopsies applying in situ immunohistochemistry at day 6 post-infection (100× magnification, scale bar 100 µm).

Abbreviations

ATP: adenosine triphosphate; CARVA: carvacrol; CBA: Cytometric Bead Array; CFU: colony forming units; HPF: high power field; IFN: interferon; IL: interleukin; LOS: lipooligosaccharide; LPS: lipopolysaccharide; MCP-1: monocyte chemoattractant protein-1; MIC: minimal inhibitory concentration; MLN: mesenteric lymph nodes; NO: nitric oxide; PBS: phosphate-buffered saline; PLC: placebo; p.i.: post-infection; SPF: specific pathogen free; TLR: Toll-like Receptor; TNF: tumor necrosis factor.

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Authors' contributions
SM: Performed experiments, co-wrote paper. AMS, CK, EL: Performed experiments, analyzed data. UE: Performed experiments. SK: Performed experiments, analyzed data, co-edited paper. SB: Provided advice in experimental design, critically discussed results, co-edited paper. MMH: Designed and performed experiments, analyzed data, wrote paper. All authors read and approved the final manuscript.

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Availability of data and materials
Not applicable.

Ethics approval and consent to participate
In vivo experiments were conducted in accordance with the European Guidelines for animal welfare (2010/63/EU) after approval by the commission for animal experiments headed by the "Landesamt für Gesundheit und Soziales" (LaGeSo, Berlin, registration numbers G0172/16 and G0247/16). Clinical conditions of mice were assessed twice a day.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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References

1. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, et al. Foodborne illness acquired in the United States—major pathogens. Emerg Infect Dis. 2011;17(1):7–15.
2. Jantsch J, Chikkaballi D, Hensel M. Cellular aspects of immunity to intracellular *Salmonella enterica*. Immunol Rev. 2011;240(1):185–95.
3. Humphrey T, O'Brien S, Madsen M. Campylobacters as zoonotic pathogens: a food production perspective. Int J Food Microbiol. 2007;117(3):237–57.
4. Možina SS, Kurinčič M, Klančnik A, Mavri A. Campylobacter and its multiresistance in the food chain. Trends Food Sci Technol. 2011;22(2):91–8.
5. Luangtongkum T, Jeon B, Han J, Plummer P, Logue CM, Zhang Q. Antibiotic resistance in Campylobacter: emergence, transmission and persistence. Future Microbiol. 2009;4(2):189–200.
6. Altekruse SF, Tollefson LK. Human campylobacteriosis: a challenge for the veterinary profession. J Am Vet Med Assoc. 2003;223(4):445–52.
7. Kist M, Bereswill S. *Campylobacter jejuni*. Contrib Microbiol. 2001;8:150–65.
8. Walker RI, Caldwell MB, Lee EC, Guerry P, Trust TJ, Ruiz-Palacios GM. Pathophysiology of *Campylobacter enteritis*. Microbiol Rev. 1986;50(1):81–94.
9. Backert S, Tegtmeyer N, Croinin TO, Boehm M, Heimesaat MM. Human campylobacteriosis. Campylobacter: features, detection, and prevention of foodborne disease. 2017. p. 1–25.
10. Nachamkin I. Chronic effects of Campylobacter infection. Microbes Infect. 2002;4(4):399–403.
11. Allos BM. Association between Campylobacter infection and Guillain-Barre syndrome. J Infect Dis. 1997;176(Suppl 2):S125–S128128.
12. Haag LM, Fischer A, Otto B, Plickert R, Kuhl AA, Gobel UB, et al. *Campylobacter jejuni* induces acute enterocolitis in gnotobiotic IL-10$^{-/-}$ mice via Toll-like-receptor-2 and -4 signaling. PLoS ONE. 2012;7(7):e40761.
13. Bereswill S, Fischer A, Plickert R, Haag LM, Otto B, Kuhl AA, et al. Novel murine infection models provide deep insights into the "menage a trois" of *Campylobacter jejuni*, microbiota and host innate immunity. PLoS ONE. 2011;6(6):e20953.
14. John CM, Phillips NJ, Stein DC, Jarvis GA. Innate immune response to lipooligosaccharide: pivotal regulator of the pathobiology of invasive *Neisseria meningitidis* infections. Pathog Dis. 2017;75(3):ftx030.
15. Mandrell RE, Apicella MA. Lipo-oligosaccharides (LOS) of mucosal pathogens: molecular mimicry and host-modification of LOS. Immunobiology. 1993;187(3–5):382–402.
16. Gutierrez-Del-Rio I, Fernandez J, Lombo F. Plant nutraceuticals as antimicrobial agents in food preservation: terpenoids, polyphenols and thiols. Int J Atimicrob Agents. 2018;52(3):309–15.
17. Suntres ZE, Coccimiglio J, Alipour M. The bioactivity and toxicological actions of carvacrol. Crit Rev Food Sci Nutr. 2015;55(3):304–18.
18. Friedman M. Chemistry and multibeneficial bioactivities of carvacrol (4-isopropyl-2-methylphenol), a component of essential oils produced by aromatic plants and spices. J Agric Food Chem. 2014;62(31):7652–70.
19. Di Pasqua R, Betts G, Hoskins N, Edwards M, Ercolini D, Mauriello G. Membrane toxicity of antimicrobial compounds from essential oils. J Agric Food Chem. 2007;55(12):4863–70.
20. Di Pasqua R, Hoskins N, Betts G, Mauriello G. Changes in membrane fatty acids composition of microbial cells induced by addiction of thymol, carvacrol, limonene, cinnamaldehyde, and eugenol in the growing media. J Agric Food Chem. 2006;54(7):2745–9.
21. Helander IM, Alakomi H-L, Latva-Kala K, Mattila-Sandholm T, Pol I, Smid EJ, et al. Characterization of the action of selected essential oil components on gram-negative bacteria. J Agric Food Chem. 1998;46(9):3590–5.
22. Gill AO, Holley RA. Inhibition of membrane bound ATPases of *Escherichia coli* and *Listeria monocytogenes* by plant oil aromatics. Int J Food Microbiol. 2006;111(2):170–4.
23. Gill AO, Holley RA. Disruption of *Escherichia coli*, *Listeria monocytogenes* and *Latobacillus sakei* cellular membranes by plant oil aromatics. Int J Food Microbiol. 2006;108(1):1–9.
24. Ultee A, Kets EP, Smid EJ. Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*. Appl Environ Microbiol. 1999;65(10):4606–10.
25. Allaoua M, Etienne P, Noirot V, Carayon JL, Tene N, Bonnafe E, et al. Pharmacokinetic and antimicrobial activity of a new carvacrol-based product against a human pathogen *Campylobacter jejuni*. J Appl Microbiol. 2018;125(4):1162–74.
26. Kelly C, Gundogdu O, Pircalabioru G, Cean A, Scates P, Linton M, et al. The in vitro and in vivo effect of carvacrol in preventing campylobacter infection, colonization and in improving productivity of chicken broilers. Foodborne Pathog Dis. 2017;14(6):341–9.
27. Arsi K, Donoghue AM, Venkitanarayanan K, Kollanoor-Johny A, Fanatico AC, Blore PJ, et al. The efficacy of the natural plant extracts, thymol and carvacrol against campylobacter colonization in broiler chickens. J Food Safety. 2014;34(4):321–5.
28. Kollanoor Johny A, Darre MJ, Donoghue AM, Donoghue DJ, Venkitanarayanan K. Antibacterial effect of trans-cinnamaldehyde, eugenol, carvacrol, and thymol on *Salmonella enteritidis* and *Campylobacter jejuni* in chicken cecal contents in vitro. J Appl Poult Res. 2010;19(3):237–44.
29. Upadhyay A, Arsi K, Wagle BR, Upadhyaya I, Shrestha S, Donoghue AM, et al. Trans-cinnamaldehyde, carvacrol, and eugenol reduce *Campylobacter jejuni* colonization factors and expression of virulence genes in vitro. Front Microbiol. 2017;8:713.
30. van Alphen LB, Burt SA, Veenendaal AK, Bleumink-Pluym NM, van Putten JP. The natural antimicrobial carvacrol inhibits *Campylobacter jejuni* motility and infection of epithelial cells. PLoS ONE. 2012;7(9):e45343.

31. Heimesaat MM, Grundmann U, Alutis ME, Fischer A, Bereswill S. Absence of nucleotide-oligomerization-domain-2 is associated with less distinct disease in *Campylobacter jejuni* infected secondary abiotic IL-10 deficient mice. Front Cell Infect Microbiol. 2017;7:322.
32. Heimesaat MM, Lugert R, Fischer A, Alutis M, Kuhl AA, Zautner AE, et al. Impact of *Campylobacter jejuni* cj0268c knockout mutation on intestinal colonization, translocation, and induction of immunopathology in gnotobiotic IL-10 deficient mice. PLoS ONE. 2014;9(2):e90148.
33. Health EPoP. Scientific opinion on the risk to plant health of *Xanthomonas citri* pv. citri and *Xanthomonas citri* pv. aurantifolii for the EU territory. EFSA J. 2014;12(2):3556.
34. La Storia A, Ercolini D, Marinello F, Di Pasqua R, Villani F, Mauriello G. Atomic force microscopy analysis shows surface structure changes in carvacrol-treated bacterial cells. Res Microbiol. 2011;162(2):164–72.
35. Additives EPanel o, Feed PoSuiA, Bampidis V, Azimonti G, Bastos MdL, Christensen H, et al. Safety and efficacy of an essential oil of *Origanum vulgare* ssp. hirtum (Link) leetsw. for all poultry species. EFSA J. 2019;17(4):e05653.
36. Aumeeruddy-Elalfi Z, Ismael IS, Hosenally M, Zengin G, Mahomoodally MF. Essential oils from tropical medicinal herbs and food plants inhibit biofilm formation in vitro and are non-cytotoxic to human cells. 3 Biotech. 2018;8(9):395.
37. Ozogul Y, Kuley E, Ucar Y, Ozogul F. Antimicrobial impacts of essential oils on food borne-pathogens. Recent Pat Food Nutr Agric. 2015;7(1):53–61.
38. Moore-Neibel K, Gerber C, Patel J, Friedman M, Ravishankar S. Antimicrobial activity of lemongrass oil against *Salmonella enterica* on organic leafy greens. J Appl Microbiol. 2012;112(3):485–92.
39. Barker SC, Altman PM. A randomised, assessor blind, parallel group comparative efficacy trial of three products for the treatment of head lice in children-melaleuca oil and lavender oil, pyrethrins and piperonyl butoxide, and a "suffocation" product. BMC Dermatol. 2010;10:6.
40. Klancnik A, Guzej B, Kolar MH, Abramovic H, Mozina SS. In vitro antimicrobial and antioxidant activity of commercial rosemary extract formulations. J Food Prot. 2009;72(8):1744–52.
41. Sharifi-Rad M, Varoni EM, Iriti M, Martorell M, Setzer WN, Del Mar CM, et al. Carvacrol and human health: a comprehensive review. Phytother Res. 2018;32(9):1675–87.
42. Baser KH. Biological and pharmacological activities of carvacrol and carvacrol bearing essential oils. Curr Pharm Des. 2008;14(29):3106–19.
43. Beaubrun JJ, Addy N, Keltner Z, Farris S, Ewing L, Gopinath G, et al. Evaluation of the impact of varied carvacrol concentrations on *Salmonella* recovery in oregano and how corn oil can minimize the effect of carvacrol during preenrichment. J Food Prot. 2018;81(6):977–85.
44. Alves Coelho Trevisan D, Fiori da Silva A, Negri M, Alves de Abreu Filho B, Junior M, Patussi E, et al. Antibacterial and antibiofilm activity of carvacrol against *Salmonella enterica* serotype Typhimurium. 2018.
45. Engel JB, Heckler C, Tondo EC, Daroit DJ, da Silva Malheiros P. Antimicrobial activity of free and liposome-encapsulated thymol and carvacrol against *Salmonella* and *Staphylococcus aureus* adhered to stainless steel. Int J Food Microbiol. 2017;252:18–23.
46. Obaidat MM, Frank JF. Inactivation of *Escherichia coli* O157:H7 on the intact and damaged portions of lettuce and spinach leaves by using allyl isothiocyanate, carvacrol, and cinnamaldehyde in vapor phase. J Food Prot. 2009;72(10):2046–55.
47. Ultee A, Slump RA, Steging G, Smid EJ. Antimicrobial activity of carvacrol toward *Bacillus cereus* on rice. J Food Prot. 2000;63(5):620–4.
48. Ultee A, Smid EJ. Influence of carvacrol on growth and toxin production by *Bacillus cereus*. Int J Food Microbiol. 2001;64(3):373–8.
49. Pesavento G, Calonico C, Bilia AR, Barnabei M, Calesini F, Addona R, et al. Antibacterial activity of Oregano, Rosmarinus and Thymus essential oils against *Staphylococcus aureus* and *Listeria monocytogenes* in beef meatballs. Food Control. 2015;54:188–99.
50. Burt S. Essential oils: their antibacterial properties and potential applications in foods—a review. Int J Food Microbiol. 2004;94(3):223–53.
51. Amirghofran Z, Ahmadi H, Karimi MH, Kalantar F, Gholijani N, Malek-Hosseini Z. In vitro inhibitory effects of thymol and carvacrol on dendritic cell activation and function. Pharm Biol. 2016;54(7):1125–32.
52. Liu SD, Song MH, Yun W, Lee JH, Kim HB, Cho JH. Effect of carvacrol essential oils on immune response and inflammation-related genes expression in broilers challenged by lipopolysaccharide. Poult Sci. 2019;98(5):2026–33.
53. Feng X, Jia A. Protective effect of carvacrol on acute lung injury induced by lipopolysaccharide in mice. Inflammation. 2014;37(4):1091–101.
54. Heimesaat MM, Bereswill S, Fischer A, Fuchs D, Struck D, Niebergall J, et al. Gram-negative bacteria aggravate murine small intestinal Th1-type immunopathology following oral infection with *Toxoplasma gondii*. J Immunol. 2006;177(12):8785–95.
55. Heimesaat MM, Alutis M, Grundmann U, Fischer A, Tegtmeyer N, Bohm M, et al. The role of serine protease HtrA in acute ulcerative enterocolitis and extra-intestinal immune responses during *Campylobacter jejuni* infection of gnotobiotic IL-10 deficient mice. Front Cell Infect Microbiol. 2014;4:77.
56. Heimesaat MM, Haag LM, Fischer A, Otto B, Kuhl AA, Gobel UB, et al. Survey of extra-intestinal immune responses in asymptomatic long-term *Campylobacter jejuni*-infected mice. Eur J Microbiol Immunol (Bp). 2013;3(3):174–82.
57. Alutis ME, Grundmann U, Fischer A, Hagen U, Kuhl AA, Gobel UB, et al. The role of gelatinases in *Campylobacter jejuni* infection of gnotobiotic mice. Eur J Microbiol Immunol. 2015;5(4):256–67.
58. Alutis ME, Grundmann U, Hagen U, Fischer A, Kuhl AA, Gobel UB, et al. Matrix metalloproteinase-2 mediates intestinal immunopathogenesis in *Campylobacter jejuni*-infected infant mice. Eur J Microbiol Immunol. 2015;5(3):188–98.
59. Heimesaat MM, Nogai A, Bereswill S, Plickert R, Fischer A, Loddenkemper C, et al. MyD88/TLR9 mediated immunopathology and gut microbiota dynamics in a novel murine model of intestinal graft-versus-host disease. Gut. 2010;59(8):1079–87.
60. Bryan NS, Grisham MB. Methods to detect nitric oxide and its metabolites in biological samples. Free Radic Biol Med. 2007;43(5):645–57.

Publisher's Note

Publikation 3

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4	Annual Review of Immunology	17,080	19.900	0.019310
5	Lancet HIV	3,301	14.813	0.018090
6	IMMUNOLOGICAL REVIEWS	15,973	13.939	0.026690
7	Science Immunology	2,516	13.440	0.015070
8	TRENDS IN IMMUNOLOGY	13,037	13.422	0.025020
9	JOURNAL OF EXPERIMENTAL MEDICINE	63,562	11.743	0.067350
10	JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY	52,417	10.228	0.077410
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37	EXERCISE IMMUNOLOGY REVIEW	784	5.300	0.000770
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Vitamin D in Acute Campylobacteriosis–Results From an Intervention Study Applying a Clinical *Campylobacter jejuni* Induced Enterocolitis Model

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Human *Campylobacter* infections are progressively rising and of high socioeconomic impact. In the present preclinical intervention study we investigated anti-pathogenic, immuno-modulatory, and intestinal epithelial barrier preserving properties of vitamin D applying an acute campylobacteriosis model. Therefore, secondary abiotic IL-$10^{-/-}$ mice were perorally treated with synthetic 25-OH-cholecalciferol starting 4 days before peroral *Campylobacter jejuni* infection. Whereas, 25-OH-cholecalciferol application did not affect gastrointestinal pathogen loads, 25-OH-cholecalciferol treated mice suffered less frequently from diarrhea in the midst of infection as compared to placebo control mice. Moreover, 25-OH-cholecalciferol application dampened *C. jejuni* induced apoptotic cell responses in colonic epithelia and promoted cell-regenerative measures. At day 6 post-infection, 25-OH-cholecalciferol treated mice displayed lower numbers of colonic innate and adaptive immune cell populations as compared to placebo controls that were accompanied by lower intestinal concentrations of pro-inflammatory mediators including IL-6, MCP1, and IFN-γ. Remarkably, as compared to placebo application synthetic 25-OH-cholecalciferol treatment of *C. jejuni* infected mice resulted in lower cumulative translocation rates of viable pathogens from the inflamed intestines to extra-intestinal including systemic compartments such as the kidneys and spleen, respectively, which was accompanied by less compromised colonic epithelial barrier function in the 25-OH-cholecalciferol as compared to the placebo cohort. In conclusion, our preclinical intervention study provides evidence that peroral synthetic 25-OH-cholecalciferol application exerts inflammation-dampening effects during acute campylobacteriosis.

Keywords: vitamin D, *Campylobacter jejuni*, campylobacteriosis model, intervention study, host-pathogen interaction, acute enterocolitis, intestinal epithelial barrier function

INTRODUCTION

Campylobacter jejuni constitute major infectious bacterial agents of zoonotic enteric morbidities with increasing prevalences worldwide (1). Humans become infected via the food chain by consumption of raw or undercooked meat derived from contaminated livestock animals or by ingestion of *C. jejuni* containing surface water (2–4). Infected individuals present with symptoms of varying degree depending on the virulence of the acquired bacterial strain on one side and the host immune status on the other (1, 5–7). Some patients display rather mild symptoms including watery diarrhea, whereas others develop acute campylobacteriosis (8, 9). These severely compromised individuals complain about abdominal cramps, fever, and inflammatory bloody diarrhea (8, 9). During infection intestinal tissues are destroyed by innate immune responses and display profound histopathological inflammatory changes such as ulcerations, crypt abscesses, and increased numbers of innate and adaptive immune cells in the colonic mucosa and lamina propria (5, 8, 10, 11). The vast majority of human infections are usually self-limiting and treated (if at all) symptomatically. Only severely compromised patients with immuno-suppressive comorbidities, for instance, require hospitalization and receive antimicrobial treatment (6, 8, 9). In rare cases, however, post-infectious sequelae such as Guillain-Barré syndrome, Miller Fisher syndrome, Reiter's syndrome, and reactive arthritis might arise with a latency of weeks to months (8, 9, 12).

Despite the progressively increasing prevalences of human campylobacteriosis, cellular, and molecular events that are involved in disease development are not yet fully understood. However, previous clinical studies revealed that in humans acute *C. jejuni* induced disease courses and post-infectious sequelae such as Guillain-Barré syndrome are triggered by the pathogenic surface molecule lipooligosaccharide (LOS) causing hyper-activation of the innate immune system in the sialylated form (13). For quite a long time *in vivo* studies have been hampered by the scarcity of appropriate animal models. This is mainly because the gastrointestinal microbiota of mice mediates a strong colconization resistance to *C. jejuni* and mice are *per se* about 10,000-fold more resistant to LOS and lipopolysaccharide (LPS) as compared to humans (14). Our group has recently shown that secondary abiotic IL-10$^{-/-}$ mice in which the gut microbiota had been depleted by broad-spectrum antibiotic treatment can not only be effectively colonized by the pathogen upon peroral infection, but also develop key features of acute campylobacteriosis such as wasting and bloody diarrhea within 1 week (15). One major reason for these severe immunopathological responses mounting in acute ulcerative enterocolitis is the absence of colonization resistance and the lack of interleukin-10 (IL-10) providing murine resistance to *C. jejuni* LOS (16, 17). In consequence, *C. jejuni* infected IL-10$^{-/-}$ mice display pronounced LOS induced and Toll-like receptor-4 (TLR-4) dependent innate and adaptive immune responses that are not restricted to the intestinal tract, but can also be observed in extra-intestinal including systemic compartments (15, 18–25).

Vitamin D has primarily been known for its regulatory properties in bone metabolism due to the tight control of calcium reabsortion in the intestinal tract and in bone remodeling (26). After exposure to ultraviolet (UV) B light the steroid hormone is produced in the skin from 7-dehydroxy-cholesterol followed by hydroxylation steps in the liver and the kidneys to the biologically active forms 25-hydroxy-vitamin D and 1,25-dihydroxy-vitamin D, respectively (27). After ingestion of food or supplements, circulating 25-hydroxy-vitamin D can be utilized by many cells including immune cells and intestinal intraepithelial cells expressing the 1α-hydroxylase enzyme CYP27B, whereas 24-hydroxylase CYP24A exerts counter-regulatory properties subsequently providing local 1,25-dihydroxy-vitamin D sources in a well-balanced fashion (27).

The identification of the vitamin D receptor (VDR) on peripheral blood mononuclear cells in the 1980s first pointed to immune-related functions of vitamin D (28, 29). In fact, vitamin D has been shown to be involved in modulating both, innate and adaptive immune responses (30–33) and to exert anti-inflammatory effects (34). Furthermore, several reports underline the anti-microbial properties of vitamin D (33). For instance, vitamin D could effectively inhibit the growth of Gram-positive bacterial strains such as *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Streptococcus mutans*, but also of Gram-negative species including *Klebsiella pneumoniae* and *Escherichia coli* (35–37). In addition, the production of antimicrobial peptides such as cathelicidin and defensins are stimulated by vitamin D (38–40). Both, immune-modulatory and antimicrobial effects might be responsible for the beneficial effects of exogenous vitamin D observed in infectious morbidities caused by *Helicobacter pylori* (41) and in respiratory tract infections (42). Moreover, vitamin D has been shown to be involved in maintenance of the intestinal epithelial barrier integrity (43).

This prompted us in our present preclinical intervention study to investigate potential pathogen-lowering, immuno-modulatory, intestinal epithelial barrier preserving and hence, disease-alleviating effects of synthetic 25-OH-cholecalciferol applying a clincial model of acute campylobacterosis.

Abbreviations: CBA, Cytometric Bead Array; CFU, colony-forming units; HPF, High power fields; IFN, interferon; IL, interleukin; LOS, Lipo-oligosaccharide; LPS, Lipo-polysaccharide; MCP-1, monocyte chemoattractant protein 1; MLN, mesenteric lymph nodes; PBS, phosphate buffered saline; p.i., post-infection; PLC, Placebo; Rt, Transmural electrical resistance; SPF, specific pathogen free; Th, T helper cell; TLR, toll-like receptor; TNF, tumor necrosis factor; Treg, regulatory T cells; UV, ultraviolet; VDR, vitamin D receptor; VitD, Vitamin D.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were conducted in accordance with the European Guidelines for animal welfare (2010/63/EU) following approval by the commission for animal experiments headed by the "Landesamt für Gesundheit und Soziales" (LaGeSo, Berlin, registration numbers G0172/16 and G0247/16). Twice a day clinical conditions of mice were assessed.

Generation of Secondary Abiotic Mice

Female and male IL-10$^{-/-}$ mice (all in C57BL/6j background) were bred and reared under specific pathogen free (SPF) conditions in the same unit of the Forschungseinrichtungen

für Experimentelle Medizin (FEM, Charité–University Medicine Berlin). Three to five mice were maintained in one cage including filter tops within an experimental semi-barrier (accessible only with lab coat, overshoes, caps, and sterile gloves) under standard conditions (22–24°C room temperature, 55 ± 15% humidity, 12 h light/12 dark cycle) and had free access to autoclaved standard chow (food pellets: ssniff R/M-H, V1534-300, Sniff, Soest, Germany).

In order to assure stable gastrointestinal *C. jejuni* colonization and to override physiological colonization resistance (44), microbiota-depleted (i.e., secondary abiotic) mice were generated (44, 45). In brief, immediately post-weaning 3-week old mice were subjected to a 10-week course of broad-spectrum antibiotic treatment by adding ampicillin plus sulbactam (1 g/L; Ratiopharm, Germany), vancomycin (500 mg/L; Cell Pharm, Germany), ciprofloxacin (200 mg/L; Bayer Vital, Germany), imipenem (250 mg/L; MSD, Germany) and metronidazole (1 g/L; Fresenius, Germany) to the autoclaved drinking water (*ad libitum*) as described elsewhere (45). To assure antibiotic washout, the antibiotic cocktail was withdrawn 4 days prior infection and thus immediately before start of the vitamin D treatment.

Vitamin D Treatment

Vitamin D treatment started 4 days before *C. jejuni* infection. Therefore, synthetic 25-OH-cholecalciferol (purchased from Sigma-Aldrich, München, Germany) was dissolved in Tween 80 (0.2% v/v) and administered to mice via the autoclaved tap water (*ad libitum*). Considering a body weight of ∼25 g per mouse and a daily drinking volume of ∼5 mL, the final concentration of the synthetic 25-OH-cholecalciferol solution was 2.5 μg/mL resulting in a daily treatment dosage of 500 μg per kg body weight (equivalent to 20,000 IU per kg) (46). Hence, the applied daily vitamin D dose was far beyond the toxic doses defined for rodents (i.e., 42 mg/kg/day) (47, 48) and humans (i.e., 150 mg/kg/day) (49). Age and sex matched placebo (PLC) control mice received vehicle (i.e., Tween 80) via the drinking water (*ad libitum*).

C. jejuni Infection, Gastrointestinal Colonization, and Translocation

For infection, a stock solution of *C. jejuni* 81-176 strain that had been stored at −80°C was thawed, aliquots streaked onto karmali agar (Oxoid, Wesel, Germany) and incubated in a microaerophilic atmosphere at 37°C for 48 h. Immediately before peroral infection of mice, bacteria were harvested in sterile PBS (Oxoid) to a final inoculum of 10^9 bacterial cells. Mice (3 months of age) were perorally infected on two consecutive days (i.e., days 0 and 1). Animals were continuously maintained in a sterile environment (autoclaved food and drinking water) and handled under strict aseptic conditions to prevent from contaminations.

In order to assess gastrointestinal colonization and translocation, *C. jejuni* were quantitatively assessed in fecal samples over time post-infection (p.i.) and furthermore, in luminal samples derived from distinct parts of the gastrointestinal tract (i.e., from the stomach, duodenum, ileum, and colon) and in organ homogenates at day 6 p.i. by culture as stated elsewhere (44, 50). The detection limit of viable pathogens was ≈100 CFU per g (CFU/g). To assess *C. jejuni* bacteremia, thioglycollate enrichment broths (BD Bioscience, Germany) were inoculated with ∼200 μL cardiac blood of individual mice, incubated for 7 days at 37°C, and streaked onto respective media for further identification as described (44).

Clinical Conditions

Before and after *C. jejuni* infection the clinical conditions of mice were assessed on a daily basis by using a standardized cumulative clinical score (maximum 12 points), addressing the clinical aspect/wasting (0: normal; 1: ruffled fur; 2: less locomotion; 3: isolation; 4: severely compromised locomotion, pre-final aspect), the abundance of blood in feces (0: no blood; 2: microscopic detection of blood by the Guajac method using Haemoccult, Beckman Coulter/PCD, Germany; 4: macroscopic blood visible), and diarrhea (0: formed feces; 2: pasty feces; 4: liquid feces) as described earlier (19).

Sampling Procedures

At day 6 p.i., mice were sacrificed by isofluran inhalation (Abbott, Germany). Luminal gastrointestinal samples (from stomach, duodenum, ileum, and colon) and *ex vivo* biopsies from colon, ileum, mesenteric lymph nodes (MLN), spleen, liver, kidneys, and lungs were taken under sterile conditions. For serum cytokine measurements cardiac blood was taken. Colonic and extra-intestinal samples were collected from each mouse in parallel for microbiological, immunohistopathological, and immunological analyses. The absolute colonic and small intestinal lengths were measured with a ruler (in cm).

Immunohistochemistry

In situ immunohistochemical analyses were performed in colonic *ex vivo* biopsies that had been immediately fixed in 5% formalin and embedded in paraffin as described earlier (51–54). In brief, in order to detect apoptotic epithelial cells, proliferation epithelial cells, macrophages/monocytes, T lymphocytes, and regulatory T cells (Tregs), 5 μm thin paraffin sections of *ex vivo* biopsies were stained with primary antibodies directed against cleaved caspase 3 (Asp175, Cell Signaling, Beverly, MA, USA, 1:200), Ki67 (TEC3, Dako, Denmark, 1:100), F4/80 (# 14-4801, clone BM8, eBioscience, San Diego, CA, USA, 1:50), CD3 (#N1580, Dako, 1:10), and FOXP3 (clone FJK-165, #14-5773, eBioscience, 1:100), respectively. Positively stained cells were then examined by light microscopy (magnification 100× and 400×), and for each mouse the average number of respective positively stained cells was determined within at least six high power fields (HPF, 0.287 mm^2, 400× magnification) by a blinded independent investigator.

Inflammatory Mediator Detection in Supernatants of Intestinal and Extra-Intestinal *ex vivo* Biopsies

Colonic *ex vivo* biopsies were cut longitudinally, washed in phosphate buffered saline (PBS; Gibco, Life Technologies, UK), and strips of ∼1 cm^2 tissue and *ex vivo* biopsies derived from MLN (3–4 lymph nodes), liver, and spleen (one half) were placed in 24-flat-bottom well-culture plates (Nunc, Germany)

containing 500 µL serum-free RPMI 1640 medium (Gibco, life technologies, UK) supplemented with penicillin (100 U/mL) and streptomycin (100 µg/mL; PAA Laboratories, Germany). After 18 h at 37°C, respective culture supernatants as well as serum samples were tested for IL-6, monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor (TNF), and interferon-γ (IFN-γ) by the Mouse Inflammation Cytometric Bead Assay (CBA; BD Biosciences, Germany) on a BD FACSCanto II flow cytometer (BD Biosciences). Systemic pro-inflammatory cytokine concentrations were measured in serum samples.

Electrophysiological Measurements

Distal colonic *ex vivo* biopsies were mounted unstripped in Ussing chambers (0.049 cm^2 area). Transmural electrical resistance (Rt) was recorded under voltage clamp conditions by an automatic clamp device (CVC6, Fiebig Hard and Software, Berlin, Germany) at 37°C over 1 h. The bathing solution was composed of NaCl (113.6 mmol/L), $NaHCO_3$ (21.0 mmol/L), KCl (5.4 mmol/L), Na_2HPO_4 (2.4 mmol/L), $MgCl_2$ (1.2 mmol/L), $CaCl_2$ (1.2 mmol/L), NaH_2PO_4 (0.6 mmol/L), D(+)-glucose (10.0 mmol/L), D(+)-mannose (10.0 mmol/L), beta-hydroxybutyric acid (0.5 mmol/L), and L-glutamine (2.5 mmol/L) equilibrated with carbogen gas (pH 7.4).

Statistical Analysis

Medians and levels of significance were determined using Mann-Whitney test (GraphPad Prism v7, USA) for pairwise comparisons of not normally distributed data, and using the one-sided ANOVA with Tukey post-correction or the Kruskal-Wallis test with Dunn's post-correction for multiple comparisons as indicated. Two-sided probability (p) values ≤ 0.05 were considered significant. Experiments were performed in a blinded fashion and reproduced three times.

RESULTS

Intestinal Pathogen Loads Over Time Following Vitamin D Treatment of *C. jejuni* Infected Mice With Acute Enterocolitis

Secondary abiotic IL-$10^{-/-}$ mice were subjected to synthetic 25-OH-cholecalciferol treatment via the drinking water starting 4 days before *C. jejuni* infection. On two consecutive days, namely days 0 and 1, mice were then perorally challenged with 10^9 viable pathogens by gavage. Daily cultural analyses of fecal samples revealed that 25-OH-cholecalciferol application did not affect pathogenic intestinal colonization properties as indicated by stable median fecal *C. jejuni* loads of 10^9 CFU/g over time p.i. that did not differ between both cohorts at respective time points (n.s.; **Figure S1**). Upon necropsy, luminal gastrointestinal *C. jejuni* densities did not differ between 25-OH-cholecalciferol and placebo treated mice as determined in stomach, duodenum, ileum and colon at day 6 post-infection (n.s.; **Figure 1**). Hence, synthetic 25-OH-cholecalciferol treatment did not affect gastrointestinal *C. jejuni* loads.

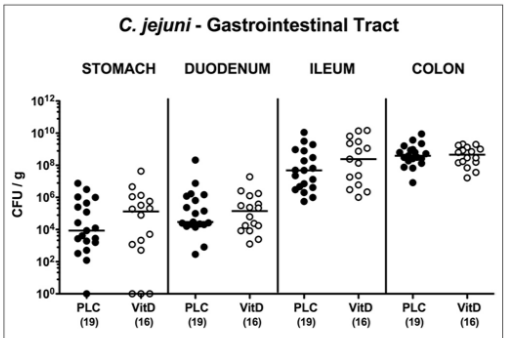

FIGURE 1 | Gastrointestinal *C. jejuni* loads following vitamin D treatment of infected mice. Secondary abiotic IL-$10^{-/-}$ mice were treated with synthetic 25-OH-cholecalciferol (vitamin D, VitD, open circles) or placebo (PLC, closed circles) via the drinking water starting 4 days before peroral *C. jejuni* 81-176 strain infection on days 0 and 1. At necropsy (i.e., day 6 post-infection), luminal *C. jejuni* loads were quantitatively assessed from each mouse in distinct gastrointestinal compartments as indicated by culture and expressed in colony forming units per g (CFU/g). Medians (black bars) and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments.

Comprehensive Survey of Clinical Conditions Over Time Following Vitamin D Treatment of *C. jejuni* Infected Mice With Acute Enterocolitis

Within 6 days following *C. jejuni* infection mice from either cohort developed comparably severe symptoms of acute enterocolitis as daily quantitated applying a standardized cumulative clinical scoring system (**Figure S2**) assessing wasting symptoms, abundance of fecal blood, and the severity of diarrhea. Whereas overall pathogen-induced clinical symptoms were comparable between the two cohorts over time (n.s.; **Figure S2**), cumulate relative frequencies of diarrhea were lower in 25-OH-cholecalciferol treated mice as compared to placebo controls as early as 24 h following the latest infection (i.e., day 2 p.i.) until 4 p.i. (**Figure 2**). Hence, synthetic 25-OH-cholecalciferol treatment results in less frequent *C. jejuni* induced diarrhea in the midst of infection.

Macroscopic and Microscopic Inflammatory Sequelae Following Vitamin D Treatment of *C. jejuni* Infected Mice With Acute Enterocolitis

Given that intestinal inflammation is association with a significant shortening of the affected part of the intestinal tract (15, 45), we measured the lengths of both, the small and large intestines upon necropsy. In fact, *C. jejuni* infection was accompanied with shorter colons of placebo as well as of 25-OH-cholecalciferol treated mice ($p < 0.001$; **Figure S3A**), whereas the small intestinal lengths were virtually unaffected at day 6 p.i. (n.s.;

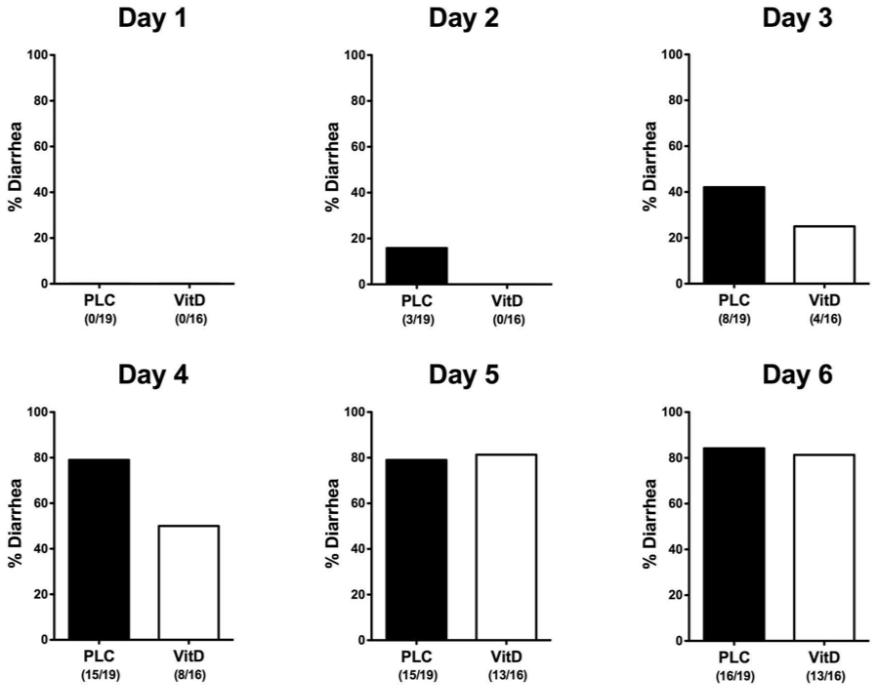

FIGURE 2 | Diarrhea frequencies over time following vitamin D treatment of *C. jejuni* infected mice. Secondary abiotic IL-10$^{-/-}$ mice were treated with synthetic 25-OH-cholecalciferol (vitamin D, VitD, white bars) or placebo (PLC, black bars) via the drinking water starting 4 days before peroral *C. jejuni* 81-176 strain infection on days 0 and 1. Occurrence of diarrhea was assessed in each mouse from day 0 until day 6 post-infection as indicated applying a standardized clinical scoring system (see Materials and Methods). Bars indicate the cumulative frequencies of diarrhea (in %). Numbers of diarrheal mice out of the total number of analyzed animals are given in parentheses. Data were pooled from four independent experiments.

Figure S3B). Hence, synthetic 25-OH-cholecalciferol treatment does not ameliorate *C. jejuni* induced macroscopic disease.

Since apoptosis is regarded a reliable parameter for the grading of intestinal inflammation (44), we further quantitatively assessed caspase3$^+$ apoptotic epithelial cells in large intestinal *ex vivo* biopsies applying *in situ* immunohistochemistry. At day 6 p.i., *C. jejuni* infected mice exhibited multifold increased numbers of apoptotic cells in their colonic epithelia ($p < 0.001$), that were, however, more than 60% lower in 25-OH-cholecalciferol as compared to placebo treated mice ($p < 0.05$; **Figure 3A**, **Figure S4A**). Conversely, numbers of Ki67$^+$ colonic epithelial cells indicative for cell proliferation and regeneration increased upon *C. jejuni* infection ($p < 0.001$), but more distinctly following 25-OH-cholecalciferol as compared to placebo treatment ($p < 0.05$; **Figure 3B**, **Figure S4B**). Hence, synthetic 25-OH-cholecalciferol treatment dampens *C. jejuni* induced apoptotic cell responses and promotes cell regenerative measures counteracting intestinal cell damage upon pathogenic exposure.

Intestinal Immune Cell Responses Following Vitamin D Treatment of *C. jejuni* Infected Mice With Acute Enterocolitis

We further quantitatively surveyed both, innate and adaptive immune cell responses in the large intestinal tract following synthetic 25-OH-cholecalciferol treatment of *C. jejuni* infected mice by immunohistochemical staining of colonic paraffin sections. As early as 6 days upon *C. jejuni* infection, numbers of F4/80$^+$ innate immune cell subsets including macrophages and monocytes had increased in the large intestinal mucosa and lamina propria ($p < 0.001$), but less distinctly in 25-OH-cholecalciferol as compared to placebo challenged mice ($p < 0.01$; **Figure 4A**, **Figure S4C**). Similarly, *C. jejuni* induced increases in adaptive immune cells such as CD3$^+$ lymphocytes, were less pronounced in the 25-OH-cholecalciferol vs. placebo cohort at day 6 p.i. ($p < 0.05$, VitD vs. PLC; **Figure 4B**, **Figure S4D**). Interestingly, numbers of FOXP3$^+$ regulatory T cells (Treg) were slightly higher following vitamin D as

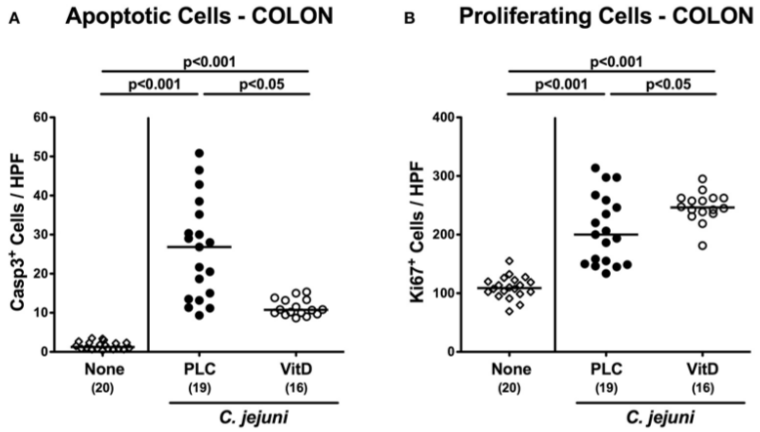

FIGURE 3 | Colonic epithelial cell apoptosis and cell proliferation/regeneration following vitamin D treatment of *C. jejuni* infected mice. Secondary abiotic IL-10$^{-/-}$ mice were treated with synthetic 25-OH-cholecalciferol (vitamin D, VitD, open circles) or placebo (PLC, closed circles) via the drinking water starting 4 days before peroral *C. jejuni* 81-176 strain infection on days 0 and 1. At necropsy (i.e., day 6 post-infection), the average numbers of colonic epithelial **(A)** apoptotic (Casp3^{+}) and **(B)** proliferating (Ki67^{+}) cells were assessed microscopically from six high power fields (HPF, 400× magnification) per animal in immunohistochemically stained colonic paraffin sections. Uninfected and untreated mice (none, open diamonds) served as negative control animals. Medians (black bars), levels of significance (*p*-values) assessed by the Kruskal-Wallis test and Dunn's post-correction or the one-sided ANOVA test with Tukey post-correction and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments.

compared to placebo treated *C. jejuni* infected mice ($p < 0.01$; **Figure 4C**, **Figure S4E**). Hence, synthetic 25-OH-cholecalciferol treatment results in less pronounced *C. jejuni* induced intestinal responses of distinct innate and adaptive immune cell populations.

Intestinal Pro-inflammatory Mediator Secretion Following Vitamin D Treatment of *C. jejuni* Infected Mice With Acute Enterocolitis

We next measured pro-inflammatory mediators in intestinal *ex vivo* biopsies. At day 6 following *C. jejuni* infection increased IL-6 and MCP-1 concentrations could be assessed in the colon of placebo ($p < 0.01$ and $p < 0.05$, respectively), but not 25-OH-cholecalciferol treated mice (**Figures 5A,B**). *C. jejuni* induced increases in large intestinal TNF and IFN-γ concentrations ($p < 0.05$–0.001 vs. none), however, were unaffected by 25-OH-cholecalciferol challenge (n.s. vs. PLC; **Figures 5C,D**). In line, ileal IL-6 and MCP-1 as well as IFN-γ levels were elevated upon *C. jejuni* infection of mice from the placebo ($p < 0.05$–0.01), but not from the 25-OH-cholecalciferol cohort (**Figures 6A,B,D**), whereas like in the colon, ileal TNF concentrations were comparably elevated at day 6 post-infection of either cohort ($p < 0.001$; **Figure 6C**). Hence, synthetic 25-OH-cholecalciferol treatment of *C. jejuni* infected mice results in less pronounced secretion of distinct pro-inflammatory mediators in the intestinal tract.

Extra-Intestinal Inflammatory Immune Responses Following Vitamin D Treatment of *C. jejuni* Infected Mice

We further asked whether the 25-OH-cholecalciferol mediated anti-inflammatory effects were restricted to the intestinal tract or also effective in extra-intestinal compartments. In fact, IFN-γ concentrations were lower in MLN and liver of 25-OH-cholecalciferol as compared to placebo treated mice at day 6 p.i. ($p < 0.05$; **Figures 7A,B**). Interestingly, *C. jejuni* infection resulted in decreased IFN-γ secretion in splenic *ex vivo* biopsies irrespective of the treatment regimen ($p < 0.001$; **Figure 7C**). Hence, synthetic 25-OH-cholecalciferol treatment of *C. jejuni* infected mice resulted in less distinct IFN-γ secretion in MLN and liver.

Systemic Pro-inflammatory Mediator Secretion Following Vitamin D Treatment of *C. jejuni* Infected Mice With Acute Enterocolitis

We next addressed whether synthetic 25-OH-cholecalciferol treatment might alleviate systemic *C. jejuni* induced pro-inflammatory immune responses. At day 6 p.i., mice from either cohort exhibited comparably elevated IL-6, MCP1, TNF, and IFN-γ serum concentrations ($p < 0.001$ vs. none; **Figure S5**). Hence, synthetic 25-OH-cholecalciferol treatment does not affect *C. jejuni* induced systemic pro-inflammatory mediator secretion.

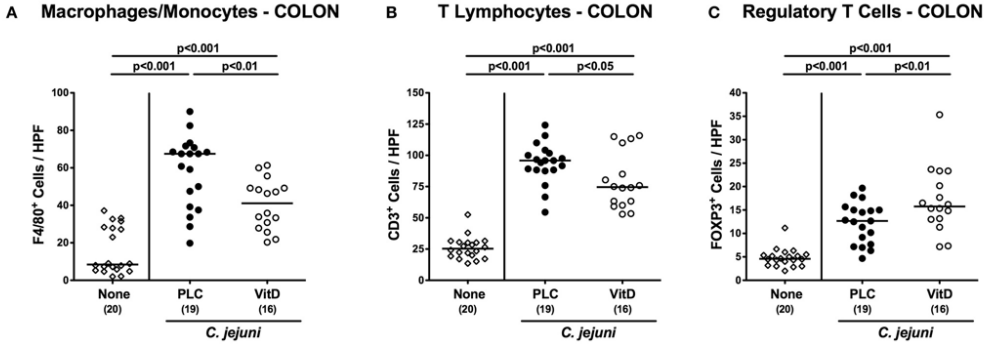

FIGURE 4 | Colonic immune cell responses following vitamin D treatment of *C. jejuni* infected mice. Secondary abiotic IL-10$^{-/-}$ mice were treated with synthetic 25-OH-cholecalciferol (vitamin D, VitD, open circles) or placebo (PLC, closed circles) via the drinking water starting 4 days before peroral *C. jejuni* 81-176 strain infection on days 0 and 1. At necropsy (i.e., day 6 post-infection), the average numbers of **(A)** macrophages and monocytes (F4/80^{+}), **(B)** T lymphocytes (CD3^{+}) and **(C)** regulatory T cells (FOXP3+) were assessed microscopically from six high power fields (HPF, 400× magnification) per animal in immunohistochemically stained colonic paraffin sections. Uninfected and untreated mice (none, open diamonds) served as negative control animals. Medians (black bars), levels of significance (*p*-values) assessed by the one-sided ANOVA test with Tukey post-correction and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments.

Bacterial Translocation Following Vitamin D Treatment of *C. jejuni* Infected Mice With Acute Enterocolitis

We further asked whether synthetic 25-OH-cholecalciferol treatment had an impact of the translocation rates of viable pathogens from the infected intestines to extra-intestinal including systemic tissue sites. Whereas, *C. jejuni* could be cultured at similar frequencies from MLN, liver and lungs derived from 25-OH-cholecalciferol and placebo treated mice (**Figures 8A–C**), cumulative pathogenic translocation rates were lower in the kidneys (12.5 vs. 31.6%) and the spleen (12.5 vs. 26.3%) taken from the former as compared to the latter at day 6 p.i. (**Figures 8D,E**). Notably, all blood cultures remained *C. jejuni* negative (**Figure 8F**). Hence, synthetic 25-OH-cholecalciferol treatment was associated with lower cumulative translocation rates of *C. jejuni* originating from the inflamed intestines to the kidneys and the spleen.

Colonic Epithelial Barrier Changes Following Vitamin D Treatment of *C. jejuni* Infected Mice With Acute Enterocolitis

Given the lower cumulative pathogenic translocation rates we assessed whether synthetic 25-OH-cholecalciferol treatment resulted in a less compromised colonic epithelial barrier function in *C. jejuni* infected mice. Therefore, we performed electrophysiological resistance measurements of colonic *ex vivo* biopsies in the Ussing chamber. In fact, transmural resistances were lower in the large intestines derived from placebo, but not 25-OH-cholecalciferol treated mice at day 6 p.i. as compared to uninfected and untreated control animals ($p < 0.05$; **Figure 9**). Hence, synthetic 25-OH-cholecalciferol treatment results in uncompromised colonic epithelial barrier function following *C. jejuni* infection.

DISCUSSION

Due to the pleiotropic beneficial effects of vitamin D in health and disease, the application of vitamin D as safe dietary supplement is currently discussed as promising option for the adjunct treatment and prophylaxis of various immunopathological morbidities including infectious diseases, intestinal inflammatory conditions, and cancer, for instance (33, 55). In our present vitamin D intervention study applying a clinical acute campylobacteriosis model, prophylactic synthetic 25-OH-cholecalciferol application starting 4 days prior murine infection resulted in dampened *C. jejuni* induced intestinal and extra-intestinal inflammatory sequalae, but could not lower the high intestinal pathogen loads of more than 10^9 viable *C. jejuni* per g feces. In support, recent reports revealed that the beneficial effects of vitamin D during gastrointestinal infection with distinct bacterial species such as *Salmonella* (56) or *Listeria monocytogenes* (57) are rather due to the pleiotropic immuno-modulatory than direct anti-microbial properties of the steroid hormone. In addition, one needs to take into consideration, that, in contrary to humans, the expression of the antimicrobial peptide cathelicidin in mice is not regulated by vitamin D, given that in the murine cathelicidin gene promoter the vitamin D response element is missing (58, 59). This could explain our observation that external 25-OH-cholecalciferol application, even in high doses, did not reduce intestinal *C. jejuni* burdens. However, it is tempting to speculate that this could be the case in humans.

Despite the high intestinal pathogenic burdens, 25-OH-cholecalciferol treated mice suffered less frequently from diarrhea

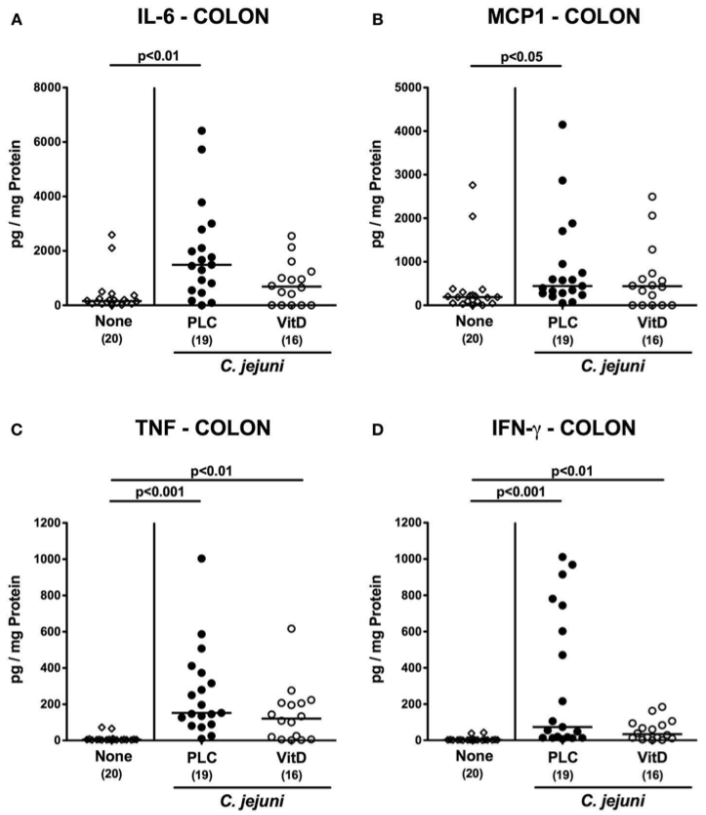

FIGURE 5 | Colonic secretion of pro-inflammatory mediators following vitamin D treatment of *C. jejuni* infected mice. Secondary abiotic IL-10$^{-/-}$ mice were treated with synthetic 25-OH-cholecalciferol (vitamin D, VitD, open circles) or placebo (PLC, closed circles) via the drinking water starting 4 days before peroral *C. jejuni* 81-176 strain infection on days 0 and 1. At necropsy (i.e., day 6 post-infection), **(A)** IL-6, **(B)** MCP1, **(C)** TNF, and **(D)** IFN-γ concentrations were determined in supernatants derived from colonic *ex vivo* biopsies. Uninfected and untreated mice (none, open diamonds) served as negative control animals. Medians (black bars), levels of significance (*p*-values) assessed by the Kruskal-Wallis test and Dunn's post-correction and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments.

in the midst of campylobacteriosis development as compared to placebo controls, but exhibited comparable macroscopic disease at the end of the observation period. Notably, the macroscopic outcome particularly in such a non-selflimiting detrimental intestinal infection and inflammation model is due to the sum effect of many different intestinal, extra-intestinal and systemic events within this hyper-inflammatory scenario (24). It is therefore remarkable, that less distinct *C. jejuni* induced apoptosis of colonic epithelial cells, whereas, conversely, large intestinal cell regenerative properties counteracting pathogen-induced cell damage were promoted upon 25-OH-cholecalciferol application in mice suffering from acute enterocolitis. In support, the intestinal epithelial vitamin D receptor has been shown to regulate mucosal inflammation by suppressing intestinal epithelial cell apoptosis (60). Less severe colonic apoptosis upon 25-OH-cholecalciferol treatment was accompanied by less distinct immune cell responses upon *C. jejuni* infection, which is supported by several studies showing that vitamin D regulates both, innate and adaptive immunity (61–63). In our study, lower numbers of innate immune cell populations such as macrophages and monocytes could be assessed in the colonic mucosa and lamina propria of *C. jejuni* infected mice that had been pretreated with synthetic 25-OH-cholecalciferol. In line, recent reports revealed that vitamin D stimulation of antigen presenting cells including macrophages and dendritic cells resulted in decreased pro-inflammatory mediator secretion

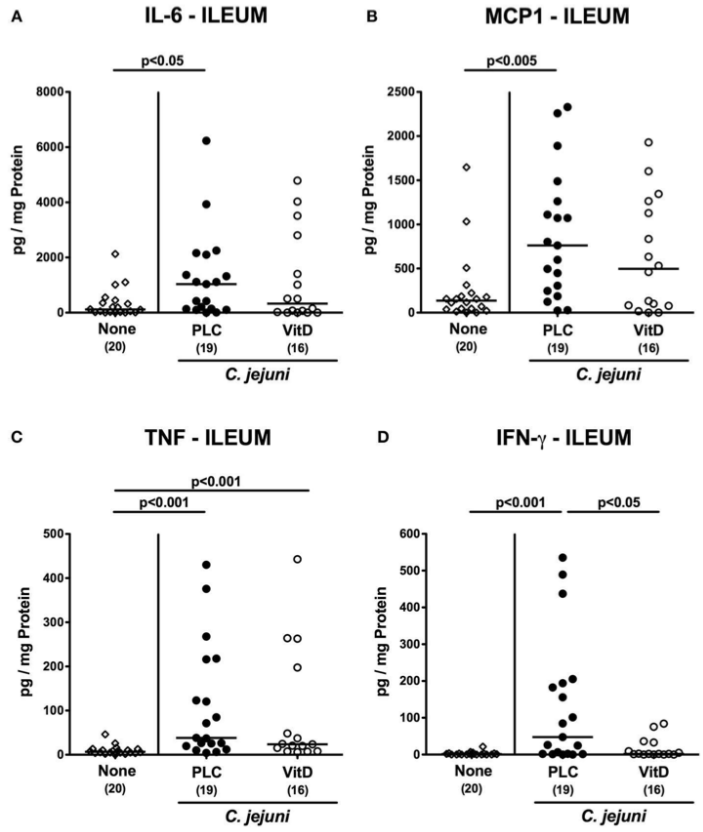

FIGURE 6 | Ileal secretion of pro-inflammatory mediators following vitamin D treatment of *C. jejuni* infected mice. Secondary abiotic IL-10$^{-/-}$ mice were treated with synthetic 25-OH-cholecalciferol (vitamin D, VitD, open circles) or placebo (PLC, closed circles) via the drinking water starting 4 days before peroral *C. jejuni* 81-176 strain infection on days 0 and 1. At necropsy (i.e., day 6 post-infection), **(A)** IL-6, **(B)** MCP1, **(C)** TNF, and **(D)** IFN-γ concentrations were determined in supernatants derived from ileal *ex vivo* biopsies. Uninfected and untreated mice (none, open diamonds) served as negative control animals. Medians (black bars), levels of significance (*p*-values) assessed by the Kruskal-Wallis test and Dunn's post-correction and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments.

(59, 64). In addition, colonic mucosal numbers of T lymphocytes were lower in 25-OH-cholecalciferol as compared to placebo treated mice with *C. jejuni* induced enterocolitis. In fact, T cells have been shown to be direct and indirect targets of vitamin D (65, 66). Previous *in vitro*, *ex vivo*, and *in vivo* studies revealed that vitamin D treatment of T cells and of mice resulted in less distinct T cell proliferation and in decreased T helper cell (Th)−1 dependent secretion of pro-inflammatory cytokines and subsequently in ameliorated inflammation (66, 67). In our present study, the colonic concentrations of pro-inflammatory mediators including IL-6 and MPC-1 measured in 25-OH-cholecalciferol pretreated, *C. jejuni* infected mice were comparable to those obtained from naive controls. In support, vitamin D was shown to reduce recruitment of innate immune cells such as monocytes and to decrease IL-6 and MCP-1 releases upon *in vitro* stimulation (68). Notably, the 25-OH-cholecalciferol associated decreased pro-inflammatry mediator secretion was not restricted to the large intestines, the major predilection site of *C. jejuni* induced enterocolitis (15, 69). In fact, *C. jejuni* induced increased secretion of IL-6, MCP-1, and additionally of IFN-γ could be observed in the terminal ileum of mice from the placebo, but not from the 25-OH-cholecalciferol treatment cohort. Interestingly, as opposed to 25-OH-cholecalciferol related decreases in large intestinal T cell

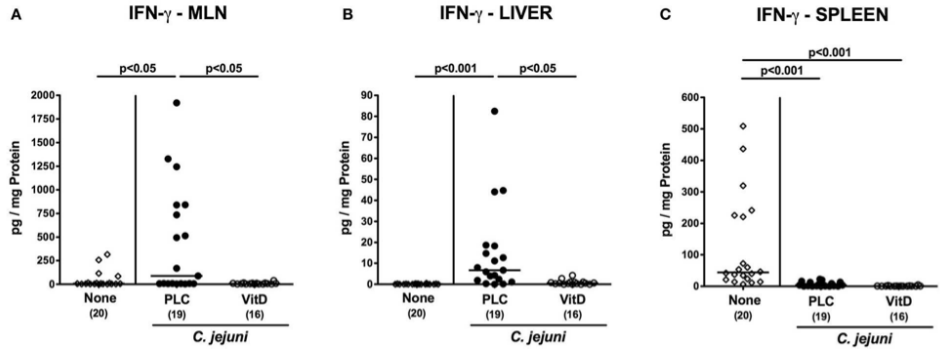

FIGURE 7 | Extra-intestinal IFN-γ secretion following vitamin D treatment of *C. jejuni* infected mice. Secondary abiotic IL-10$^{-/-}$ mice were treated with synthetic 25-OH-cholecalciferol (vitamin D, VitD, open circles) or placebo (PLC, closed circles) via the drinking water starting 4 days before peroral *C. jejuni* 81-176 strain infection on days 0 and 1. At necropsy (i.e., day 6 post-infection), IFN-γ concentrations were determined in supernatants of *ex vivo* biopsies derived from **(A)** mesenteric lymph nodes (MLN), **(B)** liver, and **(C)** spleen. Uninfected and untreated mice (none, open diamonds) served as negative control animals. Medians (black bars), levels of significance (*p*-values) assessed by the Kruskal-Wallis test and Dunn's post-correction and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments.

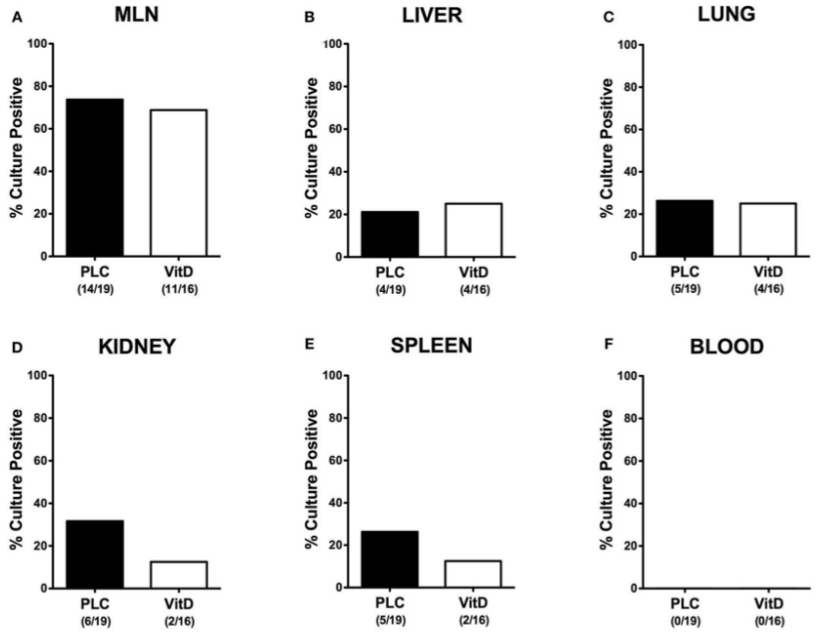

FIGURE 8 | Bacterial translocation following vitamin D treatment of *C. jejuni* infected mice. Secondary abiotic IL-10$^{-/-}$ mice were treated with synthetic 25-OH-cholecalciferol (vitamin D, VitD, white bars) or placebo (PLC, black bars) via the drinking water starting 4 days before peroral *C. jejuni* 81-176 strain infection on days 0 and 1. Upon necropsy (at day 6 post-infection), the abundance of viable pathogens was assessed in *ex vivo* biopsies taken from **(A)** mesenteric lymph nodes (MLN), **(B)** liver, **(C)** lung, **(D)** kidney, **(E)** spleen, and **(F)** cardiac blood by culture. The cumulative relative translocation rates of *C. jejuni* into the respective compartment out of four independent experiments are indicated in %. The numbers of culture-positive mice out of the total numbers of analyzed animals are given in parentheses.

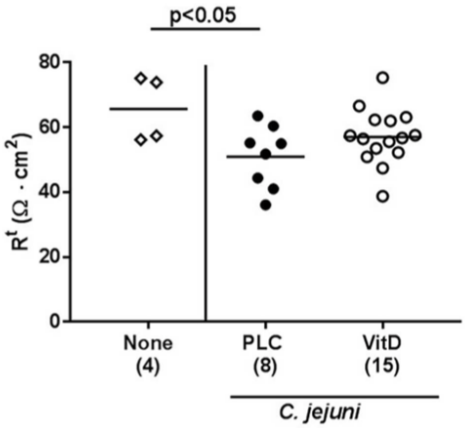

FIGURE 9 | Colonic transmural electrical resistance following vitamin D treatment of *C. jejuni* infected mice. Secondary abiotic IL-$10^{-/-}$ mice were treated with synthetic 25-OH-cholecalciferol (vitamin D, VitD, open circles) or placebo (PLC, closed circles) via the drinking water starting 4 days before peroral *C. jejuni* 81-176 strain infection on days 0 and 1. At necropsy (i.e., day 6 post-infection), the transmural electrical resistance of distal colon was measured in Ussing chambers as described in Materials and Methods. Uninfected and untreated mice (none, open diamonds) served as negative control animals. Medians (black bars), levels of significance (*p*-values) assessed by the Kruskal-Wallis test and Dunn's post-correction and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from two independent experiments.

numbers, higher numbers of (potentially anti-inflammatory) $FOXP3^+$ regulatory T cells could be assessed in the colonic mucosa and lamina propria of 25-OH-cholecalciferol vs. placebo treated mice with enterocolitis. In support, recent studies reported that vitamin D results in enhanced recruitment of regulatory T cells to inflamed tissue sites (70–72). Given that we did not perform co-staining analyses in our present study, however, we can not answer which specific immune cell subset was expressing FOXP3.

Remarkably, the pro-inflammatory immune response-dampening effects of exogenous 25-OH-cholecalciferol were not restricted to the intestinal tract, but were also effective in extra-intestinal compartments given that *C. jejuni* induced IFN-γ secretion was less pronounced in MLN draining the inflamed intestines and in the liver upon 25-OH-cholecalciferol treatment. In line, previous studies provide evidence that vitamin D application or even skin exposure to UV light could ameliorate or prevent from liver inflammation due to vitamin D mediated dampening of immune cellular responses and inhibition of liver apoptosis, for instance (73, 74).

At the first glance unexpectedly, *C. jejuni* infection was associated with decreases in splenic IFN-γ concentrations in either cohort. One possible explanation might be that upon pathogenic infection leukocytes were recruited from the spleen to the site of infection in order to limit pathogenic spread. One could have expected an even more prominent effect following synthetic 25-OH-cholecalciferol application due to the known immune cell recruiting properties of vitamin D (75).

C. jejuni infection results in impaired epithelial barrier function *in vitro* (76) and campylobacteriosis is characterized by a leaky gut syndrome facilitating pathogenic translocation from the inflamed intestines to extra-intestinal including systemic compartments (15, 69). Given that vitamin D has been shown to preserve epithelial barrier function (75), we assessed potential 25-OH-cholecalciferol mediated effects on pathogenic translocation frequencies in our preclinical survey. In fact, when taking results of the four independent experiment together, *C. jejuni* could be cultured less frequently from the kidneys and the spleen of infected mice following 25-OH-cholecalciferol as compared to placebo treatment, whereas cumulative relative *C. jejuni* translocation rates to MLN, liver and lungs were comparable. Of note, all blood cultures remained *C. jejuni* negative, irrespective of the treatment regimen. One needs to take into consideration, however, that soluble bacterial molecules including LOS and others might have been transported via the circulation contributing to the observed extra-intestinal collateral damages of *C. jejuni* infection. Nevertheless, the observed inflammation-alleviating effects upon 25-OH-cholecalciferol application were further accompanied by a less compromised colonic epithelial barrier function in 25-OH-cholecalciferol as compared to placebo treated, *C. jejuni* infected mice. This in turn very likely reduced the risk of spread of both, viable bacteria and soluble bacterial molecules in the former vs. the latter. We therefore hypothesize that the 25-OH-cholecalciferol associated anti-inflammatory effects in particular prevent from further bacteria-induced damages in this acute *C. jejuni* induced inflammation model.

CONCLUSION

Our preclinical intervention study provides evidence that prophylactic peroral synthetic 25-OH-cholecalciferol application dampens intestinal and extra-intestinal inflammatory responses during acute campylobacteriosis in the clinical mouse model applied here. Further studies are needed in order to define appropriate vitamin D doses for the prevention and combat of distinct gastrointestinal infectious morbidities in humans.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

AUTHOR CONTRIBUTIONS

SM performed experiments, analyzed data, and co-wrote paper. FL and RB performed experiments, analyzed data, and co-edited paper. J-DS and SB provided advice in experimental design, critically discussed results, and co-edited paper. MH designed and performed experiments, analyzed data, and wrote paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02094/full#supplementary-material

Figure S1 | Fecal *C. jejuni* loads over time following vitamin D treatment of infected mice. Secondary abiotic IL-10$^{-/-}$ mice were treated with **(A)** placebo (closed circles) or **(B)** synthetic 25-OH-cholecalciferol (vitamin D, open circles) via the drinking water starting four days before peroral *C. jejuni* 81-176 strain infection on day (d) 0 and d1. Fecal *C. jejuni* loads were quantitatively assessed from each mouse on a daily basis post-infection (p.i.) by culture and expressed in colony forming units per g (CFU/g). Medians (black bars) and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments.

Figure S2 | Kinetic survey of overall clinical conditions following vitamin D treatment of *C. jejuni* infected mice. Secondary abiotic IL-10$^{-/-}$ mice were treated with **(A)** placebo (closed circles) or **(B)** synthetic 25-OH-cholecalciferol (vitamin D, open circles) via the drinking water starting four days before peroral *C. jejuni* 81-176 strain infection on days 0 and 1. Clinical symptoms were quantitatively assessed applying a standardized clinical scoring system from d0 until d6 post-infection (see Materials and Methods). Uninfected and untreated mice (none, open diamonds) served as negative control animals. Medians (black bars) and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments.

Figure S3 | Intestinal lengths following vitamin D treatment of *C. jejuni* infected mice. Secondary abiotic IL-10$^{-/-}$ mice were treated with synthetic 25-OH-cholecalciferol (vitamin D, VitD, open circles) or placebo (PLC, closed circles) via the drinking water starting 4 days before peroral *C. jejuni* 81-176 strain infection on days 0 and 1. At necropsy (i.e., day 6 post-infection), the absolute lengths of the **(A)** colon and **(B)** small intestines were measured with a ruler. Uninfected and untreated mice (none, open diamonds) served as negative control animals. Medians (black bars), levels of significance (*p*-values) assessed by one-sided ANOVA test with Tukey post-correction and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments.

Figure S4 | Representative photomicrographs illustrating apoptotic and proliferating epithelial as well as immune cells responses in large intestines following vitamin D treatment of *C. jejuni* infected mice. Secondary abiotic IL-10$^{-/-}$ mice were treated with synthetic 25-OH-cholecalciferol (vitamin D) or placebo via the drinking water starting 4 days before peroral *C. jejuni* 81-176 strain infection on days 0 and 1. Naive mice served as uninfected and untreated controls. Photomicrographs reepresentative for four independent experiments illustrate the average numbers of **(A)** apoptotic epithelial cells (Casp3+), **(B)** proliferating epithelial cells, **(C)** macrophages and monocytes (F4/80+), **(D)** T lymphocytes (CD3+) and **(E)** regulatory T cell (Treg, FOXP3+) in at least six high power fields (HPF) as quantitatively assessed in ileal paraffin sections applying *in situ* immunohistochemistry at day 6 post-infection (100× magnification, scale bar 100 μm).

Figure S5 | Systemic secretion of pro-inflammatory mediators following vitamin D treatment of *C. jejuni* infected mice. Secondary abiotic IL-10$^{-/-}$ mice were treated with synthetic 25-OH-cholecalciferol (vitamin D, VitD, open circles) or placebo (PLC, closed circles) via the drinking water starting 4 days before peroral *C. jejuni* 81-176 strain infection on days 0 and 1. At necropsy (i.e., day 6 post-infection), **(A)** IL-6, **(B)** MCP1, **(C)** TNF, and **(D)** IFN-γ concentrations were determined in serum samples. Uninfected and untreated mice (none, open diamonds) served as negative control animals. Medians (black bars), levels of significance (*p*-values) assessed by the Kruskal-Wallis test and Dunn's post-correction and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments.

REFERENCES

1. Young KT, Davis LM, Dirita VJ. *Campylobacter jejuni*: molecular biology and pathogenesis. *Nat Rev Microbiol.* (2007) 5:665–79. doi: 10.1038/nrmicro1718
2. Guerry P, Szymanski CM. *Campylobacter sugars* sticking out. *Trends Microbiol.* (2008) 16:428–35. doi: 10.1016/j.tim.2008.07.002
3. Lane JA, Mehra RK, Carrington SD, Hickey RM. The food glycome: a source of protection against pathogen colonization in the gastrointestinal tract. *Int J Food Microbiol.* (2010) 142:1–13. doi: 10.1016/j.ijfoodmicro.2010.05.027
4. Hermans D, Pasmans F, Messens W, Martel A, Van Immerseel F, Rasschaert G, et al. Poultry as a host for the zoonotic pathogen *Campylobacter jejuni*. *Vector Borne Zoonotic Dis.* (2012) 12:89–98. doi: 10.1089/vbz.2011.0676
5. Janssen R, Krogfelt KA, Cawthraw SA, van Pelt W, Wagenaar JA, Owen RJ. Host-pathogen interactions in *Campylobacter* infections: the host perspective. *Clin Microbiol Rev.* (2008) 21:505–18. doi: 10.1128/CMR.00055-07
6. Havelaar AH, van Pelt W, Ang CW, Wagenaar JA, van Putten JP, Gross U, et al. Immunity to *Campylobacter*: its role in risk assessment and epidemiology. *Crit Rev Microbiol.* (2009) 35:1–22. doi: 10.1080/10408410802636017
7. O'Croinin T, Backert S. Host epithelial cell invasion by *Campylobacter jejuni*: trigger or zipper mechanism? *Front Cell Infect Microbiol.* (2012) 2:25. doi: 10.3389/fcimb.2012.00025
8. Kist M, Bereswill S. *Campylobacter jejuni*. *Contrib Microbiol.* (2001) 8:150–65.
9. Backert S, Tegtmeyer N, Cróinín TÓ, Boehm M, Heimesaat MM. Chapter 1–Human campylobacteriosis. In Klein G, editor. *Campylobacter*. London: Academic Press (2017). p. 1–25.
10. van Spreeuwel JP, Duursma GC, Meijer CJ, Bax R, Rosekrans PC, Lindeman J. *Campylobacter colitis*: histological immunohistochemical and ultrastructural findings. *Gut.* (1985) 26:945–51. doi: 10.1136/gut.26.9.945
11. Walker RI, Caldwell MB, Lee EC, Guerry P, Trust TJ, Ruiz-Palacios GM. Pathophysiology of *Campylobacter enteritis*. *Microbiol Rev.* (1986) 50:81–94.
12. Allos BM. Association between *Campylobacter* infection and Guillain-Barre syndrome. *J Infect Dis.* (1997) 176(Suppl 2):S125–8. doi: 10.1086/513783
13. Mortensen NP, Kuijf ML, Ang CW, Schiellerup P, Krogfelt KA, Jacobs BC, et al. Sialylation of *Campylobacter jejuni* lipo-oligosaccharides is associated with severe gastro-enteritis and reactive arthritis. *Microbes Infect.* (2009) 11:988–94. doi: 10.1016/j.micinf.2009.07.004
14. da Silva AMT, Kaulbach HC, Chuidian FS, Lambert DR, Suffredini AF, Danner RL. Shock and multiple-organ dysfunction after self-administration of Salmonella endotoxin. *N Engl J Med.* (1993) 328:1457–60.
15. Haag LM, Fischer A, Otto B, Plickert R, Kuhl AA, Gobel UB, et al. *Campylobacter jejuni* induces acute enterocolitis in gnotobiotic IL-10$^{-/-}$

mice via Toll-like-receptor-2 and−4 signaling. *PLoS ONE*. (2012) 7:e40761. doi: 10.1371/journal.pone.0040761
16. Robertson SA, Care AS, Skinner RJ. Interleukin 10 regulates inflammatory cytokine synthesis to protect against lipopolysaccharide-induced abortion and fetal growth restriction in mice. *Biol Reprod*. (2007) 76:738–48. doi: 10.1095/biolreprod.106.056143
17. Warren HS, Fitting C, Hoff E, Adib-Conquy M, Beasley-Topliffe L, Tesini B, et al. Resilience to bacterial infection: difference between species could be due to proteins in serum. *J Infect Dis*. (2010) 201:223–32. doi: 10.1086/649557
18. Alutis ME, Grundmann U, Fischer A, Kuhl AA, Bereswill S, Heimesaat MM. Selective gelatinase inhibition reduces apoptosis and pro-inflammatory immune cell responses in *Campylobacter jejuni*-infected gnotobiotic IL-10 deficient mice. *Eur J Microbiol Immunol*. (2014) 4:213–22. doi: 10.1556/EUJMI-D-14-00031
19. Heimesaat MM, Alutis M, Grundmann U, Fischer A, Tegtmeyer N, Böhm M, et al. The role of serine protease HtrA in acute ulcerative enterocolitis and extra-intestinal immune responses during *Campylobacter jejuni* infection of gnotobiotic IL-10 deficient mice. *Front Cell Infect Microbiol*. (2014) 4:77. doi: 10.3389/fcimb.2014.00077
20. Bereswill S, Ekmekciu I, Escher U, Fiebiger U, Stingl K, Heimesaat MM. *Lactobacillus johnsonii* ameliorates intestinal, extra-intestinal and systemic pro-inflammatory immune responses following murine *Campylobacter jejuni* infection. *Sci Rep*. (2017) 7:2138. doi: 10.1038/s41598-017-02436-2
21. Bereswill S, Grundmann U, Alutis ME, Fischer A, Heimesaat MM. *Campylobacter jejuni* infection of conventionally colonized mice lacking nucleotide-oligomerization-domain-2. *Gut Pathog*. (2017) 9:5. doi: 10.1186/s13099-017-0155-3
22. Ekmekciu I, Fiebiger U, Stingl K, Bereswill S, Heimesaat MM. Amelioration of intestinal and systemic sequelae of murine *Campylobacter jejuni* infection by probiotic VSL#3 treatment. *Gut Pathog*. (2017) 9:17. doi: 10.1186/s13099-017-0168-y
23. Heimesaat MM, Grundmann U, Alutis ME, Fischer A, Bereswill S. Absence of nucleotide-oligomerization-domain-2 is associated with less distinct disease in *Campylobacter jejuni* infected secondary abiotic IL-10 deficient mice. *Front Cell Infect Microbiol*. (2017) 7:322. doi: 10.3389/fcimb.2017.00322
24. Schmidt AM, Escher U, Mousavi S, Boehm M, Backert S, Bereswill S, et al. Protease activity of *Campylobacter jejuni* HtrA modulates distinct intestinal and systemic immune responses in infected secondary abiotic IL-10 deficient mice. *Front Cell Infect Microbiol*. (2019) 9:79. doi: 10.3389/fcimb.2019.00079
25. Heimesaat MM, Grundmann U, Alutis ME, Fischer A, Bereswill S. Microbiota composition and immune responses during *Campylobacter jejuni* infection in conventionally colonized IL-10($^{-/-}$) mice lacking nucleotide oligomerization domain 2. *Eur J Microbiol Immunol*. (2017) 7:1–14. doi: 10.1556/1886.2016.00038
26. Holick MF. Vitamin D deficiency. *N Engl J Med*. (2007) 357:266–81. doi: 10.1056/NEJMra070553
27. Norman AW. From vitamin D to hormone D: fundamentals of the vitamin D endocrine system essential for good health. *Am J Clin Nutr*. (2008) 88:491s−9s. doi: 10.1093/ajcn/88.2.491S
28. Bhalla AK, Amento EP, Clemens TL, Holick MF, Krane SM. Specific high-affinity receptors for 1,25-dihydroxyvitamin D3 in human peripheral blood mononuclear cells: presence in monocytes and induction in T lymphocytes following activation. *J Clin Endocrinol Metab*. (1983) 57:1308–10. doi: 10.1210/jcem-57-6-1308
29. Provvedini DM, Tsoukas CD, Deftos LJ, Manolagas SC. 1,25-dihydroxyvitamin D3 receptors in human leukocytes. *Science*. (1983) 221:1181–3.
30. Cantorna MT. Vitamin D and its role in immunology: multiple sclerosis, and inflammatory bowel disease. *Prog Biophys Mol Biol*. (2006) 92:60–4. doi: 10.1016/j.pbiomolbio.2006.02.020
31. White JH. Vitamin D signaling, infectious diseases, and regulation of innate immunity. *Infect Immun*. (2008) 76:3837–43. doi: 10.1128/IAI.00353-08
32. Hart PH, Gorman S, Finlay-Jones JJ. Modulation of the immune system by UV radiation: more than just the effects of vitamin D? *Nat Rev Immunol*. (2011) 11:584–96. doi: 10.1038/nri3045
33. Huang FC. Vitamin D differentially regulates Salmonella-induced intestine epithelial autophagy and interleukin-1beta expression. *World J Gastroenterol*. (2016) 22:10353–63. doi: 10.3748/wjg.v22.i47.10353
34. Dauletbaev N, Herscovitch K, Das M, Chen H, Bernier J, Matouk E, et al. Down-regulation of IL-8 by high-dose vitamin D is specific to hyperinflammatory macrophages and involves mechanisms beyond up-regulation of DUSP1. *Br J Pharmacol*. (2015) 172:4757–71. doi: 10.1111/bph.13249
35. Feindt E, Stroder J. Studies on the antimicrobial effect of vitamin D. *Klin Wochenschr*. (1977) 55:507–8.
36. Youssef DA, Miller CW, El-Abbassi AM, Cutchins DC, Cutchins C, Grant WB, et al. Antimicrobial implications of vitamin D. *Dermatoendocrinol*. (2011) 3:220–9. doi: 10.4161/derm.3.4.15027
37. Saputo S, Faustoferri RC, Quivey RG Jr. Vitamin D compounds are bactericidal against streptococcus mutans and target the bacitracin-associated efflux system. *Antimicrob Agents Chemother*. (2018) 62:e01675–17. doi: 10.1128/AAC.01675-17
38. Zanetti M. Cathelicidins, multifunctional peptides of the innate immunity. *J Leukoc Biol*. (2004) 75:39–48. doi: 10.1189/jlb.0403147
39. Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR, et al. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science*. (2006) 311:1770–3. doi: 10.1126/science.1123933
40. Adams JS, Hewison M. Unexpected actions of vitamin D: new perspectives on the regulation of innate and adaptive immunity. *Nat Clin Pract Endocrinol Metab*. (2008) 4:80–90. doi: 10.1038/ncpendmet0716
41. Kawaura A, Takeda E, Tanida N, Nakagawa K, Yamamoto H, Sawada K, et al. Inhibitory effect of long term 1α-hydroxyvitamin D3 administration on Helicobacter pylori infection. *J Clin Biochem Nutr*. (2006) 38:103–6. doi: 10.3164/jcbn.38.103
42. Juzeniene A, Ma LW, Kwitniewski M, Polev GA, Lagunova Z, Dahlback A, et al. The seasonality of pandemic and non-pandemic influenzas: the roles of solar radiation and vitamin D. *Int J Infect Dis*. (2010) 14:e1099–105. doi: 10.1016/j.ijid.2010.09.002
43. Barbachano A, Fernandez-Barral A, Ferrer-Mayorga G, Costales-Carrera A, Larriba MJ, Munoz A. The endocrine vitamin D system in the gut. *Mol Cell Endocrinol*. (2017) 453:79–87. doi: 10.1016/j.mce.2016.11.028
44. Bereswill S, Fischer A, Plickert R, Haag LM, Otto B, Kuhl AA, et al. Novel murine infection models provide deep insights into the "menage a trois" of *Campylobacter jejuni*, microbiota and host innate immunity. *PLoS ONE*. (2011) 6:e20953. doi: 10.1371/journal.pone.0020953
45. Heimesaat MM, Bereswill S, Fischer A, Fuchs D, Struck D, Niebergall J, et al. Gram-negative bacteria aggravate murine small intestinal Th1-type immunopathology following oral infection with *Toxoplasma gondii*. *J Immunol*. (2006) 177:8785–95. doi: 10.4049/jimmunol.177.12.8785
46. Williamson L, Hayes A, Hanson ED, Pivonka P, Sims NA, Gooi JH. High dose dietary vitamin D3 increases bone mass and strength in mice. *Bone Rep*. (2017) 6:44–50. doi: 10.1016/j.bonr.2017.02.001
47. Gascon-Barre M, Cote MG. Effects of phenobarbital and diphenylhydantoin on acute vitamin D3 toxicity in the rat. *Toxicol Appl Pharmacol*. (1978) 43:125–35.
48. Hatch RC, Laflamme DP. Acute intraperitoneal cholecalciferol (vitamin D3) toxicosis in mice: its nature and treatment with diverse substances. *Vet Hum Toxicol*. (1989) 31:105–12.
49. Alshahrani F, Aljohani N. Vitamin D: deficiency, sufficiency and toxicity. *Nutrients*. (2013) 5:3605–16. doi: 10.3390/nu5093605
50. Heimesaat MM, Haag LM, Fischer A, Otto B, Kuhl AA, Gobel UB, et al. Survey of extra-intestinal immune responses in asymptomatic long-term *Campylobacter jejuni*-infected mice. *Eur J Microbiol Immunol*. (2013) 3:174–82. doi: 10.1556/EuJMI.3.2013.3.4
51. Heimesaat MM, Nogai A, Bereswill S, Plickert R, Fischer A, Loddenkemper C, et al. MyD88/TLR9 mediated immunopathology and gut microbiota dynamics in a novel murine model of intestinal graft-versus-host disease. *Gut*. (2010) 59:1079–87. doi: 10.1136/gut.2009.197434
52. Heimesaat MM, Lugert R, Fischer A, Alutis M, Kuhl AA, Zautner AE, et al. Impact of *Campylobacter jejuni* cj0268c knockout mutation on intestinal colonization, translocation, and induction of immunopathology in gnotobiotic IL-10 deficient mice. *PLoS ONE*. (2014) 9:e90148. doi: 10.1371/journal.pone.0090148
53. Alutis ME, Grundmann U, Fischer A, Hagen U, Kuhl AA, Gobel UB, et al. The role of gelatinases in *Campylobacter jejuni* infection of gnotobiotic mice. *Eur J Microbiol Immunol*. (2015) 5:256–67. doi: 10.1556/1886.2015.00033

54. Alutis ME, Grundmann U, Hagen U, Fischer A, Kuhl AA, Gobel UB, et al. Matrix metalloproteinase-2 mediates intestinal immunopathogenesis in *Campylobacter jejuni*-infected infant mice. *Eur J Microbiol Immunol.* (2015) 5:188–98. doi: 10.1556/1886.2015.00020
55. Nicholson I, Dalzell AM, El-Matary W. Vitamin D as a therapy for colitis: a systematic review. *J Crohns Colitis*. (2012) 6:405–11. doi: 10.1016/j.crohns.2012.01.007
56. Huang FC. The differential effects of 1,25-dihydroxyvitamin D3 on Salmonella-induced interleukin-8 and human beta-defensin-2 in intestinal epithelial cells. *Clin Exp Immunol.* (2016) 185:98–106. doi: 10.1111/cei.12792
57. Bruce D, Ooi JH, Yu S, Cantorna MT. Vitamin D and host resistance to infection? Putting the cart in front of the horse. *Exp Biol Med.* (2010) 235:921–7. doi: 10.1258/ebm.2010.010061
58. Gombart AF, Borregaard N, Koeffler HP. Human cathelicidin antimicrobial peptide. (CAMP) gene is a direct target of the vitamin D receptor and is strongly up-regulated in myeloid cells by 1,25-dihydroxyvitamin D3. *FASEB J.* (2005) 19:1067–77. doi: 10.1096/fj.04-3284com
59. Ooi JH, Li Y, Rogers CJ, Cantorna MT. Vitamin D regulates the gut microbiome and protects mice from dextran sodium sulfate-induced colitis. *J Nutr.* (2013) 143:1679–86. doi: 10.3945/jn.113.180794
60. He L, Liu T, Shi Y, Tian F, Hu H, Deb DK, et al. Gut epithelial vitamin D receptor regulates microbiota-dependent mucosal inflammation by suppressing intestinal epithelial cell apoptosis. *Endocrinology.* (2018) 159:967–79. doi: 10.1210/en.2017-00748
61. Baeke F, Takiishi T, Korf H, Gysemans C, Mathieu C. Vitamin D: modulator of the immune system. *Curr Opin Pharmacol.* (2010) 10:482–96. doi: 10.1016/j.coph.2010.04.001
62. Cantorna MT. Mechanisms underlying the effect of vitamin D on the immune system. *Proc Nutr Soc.* (2010) 69:286–9. doi: 10.1017/S0029665110001722
63. Chun RF, Liu PT, Modlin RL, Adams JS, Hewison M. Impact of vitamin D on immune function: lessons learned from genome-wide analysis. *Front Physiol.* (2014) 5:151. doi: 10.3389/fphys.2014.00151
64. Bartels LE, Bendix M, Hvas CL, Jorgensen SP, Agnholt J, Agger R, et al. Oral vitamin D3 supplementation reduces monocyte-derived dendritic cell maturation and cytokine production in Crohn's disease patients. *Inflammopharmacology.* (2014) 22:95–103. doi: 10.1007/s10787-013-0197-1
65. Cantorna MT, McDaniel K, Bora S, Chen J, James J. Vitamin D, immune regulation, the microbiota, and inflammatory bowel disease. *Exp Biol Med.* (2014) 239:1524–30. doi: 10.1177/1535370214523890
66. Cantorna MT, Waddell A. The vitamin D receptor turns off chronically activated T cells. *Ann N Y Acad Sci.* (2014) 1317:70–5. doi: 10.1111/nyas.12408
67. Kongsbak M, Levring TB, Geisler C, von Essen MR. The vitamin d receptor and T cell function. *Front Immunol.* (2013) 4:148. doi: 10.3389/fimmu.2013.00148
68. Gao D, Trayhurn P, Bing C. 1,25-Dihydroxyvitamin D3 inhibits the cytokine-induced secretion of MCP-1 and reduces monocyte recruitment by human preadipocytes. *Int J Obes.* (2013) 37:357–65. doi: 10.1038/ijo.2012.53
69. Haag LM, Fischer A, Otto B, Grundmann U, Kuhl AA, Gobel UB, et al. *Campylobacter jejuni* infection of infant mice: acute enterocolitis is followed by asymptomatic intestinal and extra-intestinal immune responses. *Eur J Microbiol Immunol.* (2012) 2:2–11. doi: 10.1556/EuJMI.2.2012.1.2
70. Unger WW, Laban S, Kleijwegt FS, van der Slik AR, Roep BO. Induction of Treg by monocyte-derived DC modulated by vitamin D3 or dexamethasone: differential role for PD-L1. *Eur J Immunol.* (2009) 39:3147–59. doi: 10.1002/eji.200839103
71. Van Belle TL, Vanherwegen AS, Feyaerts D, De Clercq P, Verstuyf A, Korf H, et al. 1,25-Dihydroxyvitamin D3 and its analog TX527 promote a stable regulatory T cell phenotype in T cells from type 1 diabetes patients. *PLoS ONE.* (2014) 9:e109194. doi: 10.1371/journal.pone.0109194
72. Christakos S, Dhawan P, Verstuyf A, Verlinden L, Carmeliet G. Vitamin D: metabolism, molecular mechanism of action, and pleiotropic effects. *Physiol Rev.* (2016) 96:365–408. doi: 10.1152/physrev.00014.2015
73. Gorman S, Black LJ, Feelisch M, Hart PH, Weller R. Can skin exposure to sunlight prevent liver inflammation? *Nutrients.* (2015) 7:3219–39. doi: 10.3390/nu7053219
74. Jahn D, Dorbath D, Kircher S, Nier A, Bergheim I, Lenaerts K, et al. Beneficial effects of vitamin D treatment in an obese mouse model of non-alcoholic steatohepatitis. *Nutrients.* (2019) 11:77. doi: 10.3390/nu11010077
75. Schwalfenberg GK. A review of the critical role of vitamin D in the functioning of the immune system and the clinical implications of vitamin D deficiency. *Mol Nutr Food Res.* (2011) 55:96–108. doi: 10.1002/mnfr.201000174
76. Bucker R, Krug SM, Moos V, Bojarski C, Schweiger MR, Kerick M, et al. *Campylobacter jejuni* impairs sodium transport and epithelial barrier function via cytokine release in human colon. *Mucosal Immunol.* (2018) 11:575–7. doi: 10.1038/mi.2017.78

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Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Master of Science (Molekular- und Zellbiologie):

durchgeführt in der AG Maurer, Klinik für Dermatologie, Venerologie und Allergologie, Charité - Campus Mitte

Thema: Erforschung der Mastzellen-Heterogenität im Hautgewebe der Maus

Mastzellen wurden in vielen Wirbeltieren, v.a. in der Maus, Ratte und in Menschen identifiziert. Schon nach ihrer Entdeckung durch Paul Ehrlich im Jahre 1878 haben die Forscher erkannt, dass Mastzellen keine einheitliche Population sind, sondern eine Heterogenität auf der Grundlage von histochemischen, Expression- und funktionellen Eigenschaften aufweisen. Mastzellen der Haut sind nicht homogen im Gewebe verteilt, sondern akkumulieren in verschieden Bereichen der Haut, in der Nähe von Blut- und lymphatischen Gefäßen, Nervenzellen, Haarfollikel und innerhalb der Basalmembran, wo ein Gradient der Mastzellen auch vermutet wird. Diese auffällige Verteilung in bestimmten Arealen der Haut lässt auf verschieden Mastzellsubpopulationen schließen, die sich aufgrund ihrer Lokalisation hinsichtlich ihres Phänotyps und Funktion unterscheiden könnten.
Die Mastzellen wurden aus den Ohren von 8-12 Wochen alten C57BL/6-Cpa3-Cre; Mcl-1+/+ sowie mastzelldefizieten C57BL/6-Cpa3-Cre; Mcl-fl/fl Mäusen isoliert und dann an einem BD FACS Aria II Zellsortierer nach der Expression der drei Mastzellenmarker CD117 (c-kit), FcεRI und CD200R3 sortiert. Durch *Whole transcriptome Amplification* synthetisierte cDNA der sortierten Zellen wurde auf *Housekeeping* Gene sowie mastzellspezifische und -unspezifische Gene mittels *touchdown-PCR* untersucht.
Durch den enzymatischen Gewebeaufschluss wurden über 15.000 Mastzellen aus der Haut isoliert. Es wurden auch aus dem Knochenmark gewonnene Mastzellen der Wildtypmaus kultiviert und als positiv Kontrolle für Genexpression eingesetzt. Die aus der Haut gewonnenen Zellen wurden an einem BD FACS Aria II zur cDNA-Synthese und Analyse sortiert. Zur Zertifizierung der Mastzellensortierung wurde die cDNA der sortierten Zellen mittels *touchdown-PCR* auf mastzellenspezifische Gene, wie z.B. *tpsb2* (Tryptase), *kit* (CD117) und *FcεRIa,* positiv untersucht. Die sortierten Zellen wurden ebenfalls auf mastzellenunspezifische Gene untersucht und wie erwartet, wurden keine PCR-Produkte dieser Gene gebildet. Außerdem wurden die sortierten Zellen mittels Pappenheimfärbung ebenso als Mastzellen identifiziert. Im Gegensatz zu den Mastzellen wurde Heterogenität bei fast allen Zellen des Immunsystems intensiv untersucht und gezeigt. Das legt die Vermutung nahe, dass auch Mastzellenheterogenität weit über die Einteilung in Schleimhautmastzellen und Bindegewebsmastzellen hinausgehen könnte. Diese Arbeit dient als Einstieg zur Beweisführung dieser These.

Bachelor of Science (Biologie):

durchgeführt in der AG Koch des Instituts für Biologie, Neurophysiologie an der Freien Universität Berlin

Thema: Die Verteilung von hyperpolarisation-aktivierten Kanälen in GABAergen Zellen des auditorischen Mittelhirns

Die auditorische Hörbahn ermöglicht die Wahrnehmung von akustischen Signalen. Die Sinneszellen, die als Mechanorezeptoren in der Hörschnecke des Innenohrs (Cochlea) liegen, nehmen diese Signale auf, wandeln sie in elektrische Nervensignale um und leiten sie über den VIII. Hirnnerv an das Zentralnervensystem weiter. Um die Informationen weiterzuleiten, werden zahlreiche Neuronen benötigt. Etwa 20% bis 30% dieser Neuronen sind inhibitorische Neuronen, die als Neurotransmittersubstanz Gamma-AminoButtersäure (GABAerg) verwenden.

Zwei wesentliche Funktionsmerkmale der hyperpolarisationsaktivierte und zyklisch nukleotidgesteuerte Ionen (HCN)-Kanäle sind die Aktivierung durch Hyperpolarisierung und die Modulation durch cAMP zur positiven Verschiebung der Aktivierungskurve. Anhand von Stromspannungskurven wurde festgestellt, dass der Hyperpolarisation-sac in den inhibitorischen Neuronen langsamer als bei anderen Neuronen verläuft. Aufgrund dieser Ergebnisse wurde die Hypothese aufgestellt, dass in den inhibitorischen Neuronen hauptsächlich HCN4-Kanäle exprimiert werden, während in exzitatorischen Neuronen vorwiegend die HCN1-Kanäle vorhanden sind.

Mithilfe von immunhistochemischen Färbungen und Aufnahmen am konfokalen Laserscanning Mikroskop wurden die GABAergen Zellen der VGAT-ChR2-YFP Maus mit Antikörpern gegen HCN1 als auch die von HCN4-Kanälen charakterisiert bzw. die Kolokalisation von HCN1- und HCN4-Kanälen im Cortex der Mäuse untersucht. Es konnte keine Kolokalisation von HCN-1 und HCN-4 beobachtet werden. Interessanterweise zeigten nur die HCN-4 Kanäle und nicht die HCN-1 Kanäle eine Koexpression mit VGAT, welches spezifisch in inhibitorischen Neuronen exprimiert wird.

Diese Ergebnisse unterstreichen die entscheidende Rolle von HCN-4 Kanäle in inhibitorischen Neuronen, indem sie die Zelle vor zu großer Hyperpolarisation schützen und somit an der Verarbeitung einströmender synaptischer Signale beteiligt sind.

Publikationsliste

1. Heimesaat, Markus M., **Soraya Mousavi**, Dennis Weschka and Stefan Bereswill. “Anti-Pathogenic and Immune-Modulatory Effects of Peroral Treatment with Cardamom Essential Oil in Acute Murine Campylobacteriosis.” Microorganisms 9 no. 1 (**2021**), 169.
2. **Mousavi, Soraya**, Stefan Bereswill, and Markus M. Heimesaat. “Novel Clinical Campylobacter jejuni Infection Models Based on Sensitization of Mice to Lipooligosaccharide, a Major Bacterial Factor Triggering Innate Immune Responses in Human Campylobacteriosis”. Microorganisms 8, no. 4 (**2020**): 482.
3. **Mousavi, Soraya**, Ulrike Escher, Elisa Thunhorst, Sophie Kittler, Corinna Kehrenberg, Stefan Bereswill, and Markus M. Heimesaat. “Vitamin C alleviates acute enterocolitis in *Campylobacter jejuni* infected mice”. Scientific reports 10, no. 1 (**2020**): 1-13.
4. **Mousavi, Soraya**, Anna-Maria Schmidt, Ulrike Escher, Sophie Kittler, Corinna Kehrenberg, Elisa Thunhorst, Stefan Bereswill, and Markus M. Heimesaat. “Carvacrol ameliorates acute campylobacteriosis in a clinical murine infection model”. Gut Pathogens 12, no. 1 (**2020**): 1-16.
5. **Mousavi, Soraya**, Dennis Weschka, Stefan Bereswill, and Markus M. Heimesaat. "Preclinical Evaluation of Oral Urolithin-A for the Treatment of Acute Campylobacteriosis in *Campylobacter jejuni* Infected Microbiota-Depleted IL-10-/-Mice." Pathogens 10, no. 1 (**2020**): 7.
6. Heimesaat, Markus M., **Soraya Mousavi**, Ulrike Escher, Fábia Daniela Lobo de Sá, Elisa Peh, Jörg-Dieter Schulzke, Sophie Kittler, Roland Bücker, and Stefan Bereswill. "Resveratrol Alleviates Acute *Campylobacter jejuni* Induced Enterocolitis in a Preclinical Murine Intervention Study." Microorganisms 8, no. 12 (**2020**): 1858.
7. Kløve, Sigri, Claudia Genger, Dennis Weschka, **Soraya Mousavi**, Stefan Bereswill, and Markus M. Heimesaat. "Toll-Like Receptor-4 Is Involved in Mediating Intestinal and Extra-Intestinal Inflammation in *Campylobacter coli*-Infected Secondary Abiotic IL-10-/-Mice." Microorganisms 8, no. 12 (**2020**): 1882.
8. Heimesaat, Markus M., **Soraya Mousavi**, Sigri Kløve, Claudia Genger, Dennis Weschka, Eliezer Giladi, Stefan Bereswill, Illana Gozes. “Immune-modulatory Properties of the Octapeptide NAP in *Campylobacter jejuni* Infected Mice Suffering from Acute Enterocolitis”. Microorganisms (**2020**), 8, 802.
9. Kløve, Sigri, Claudia Genger, **Soraya Mousavi**, Dennis Weschka, Stefan Bereswill, Markus M. Heimesaat. “Toll-Like Receptor-4 Dependent Intestinal and Systemic Sequelae Following Peroral *Campylobacter coli* Infection of IL10 Deficient Mice Harboring a Human Gut Microbiota”. Pathogens (**2020**), 9, 386.
10. Genger, C., Sigri Kløve, **Soraya Mousavi**, Stefan Bereswill, Markus M. Heimesaat. “The conundrum of colonization resistance against *Campylobacter* reloaded: the gut microbiota composition in conventional mice does not prevent from *Campylobacter coli* infection”. Eur J Microbiol Immun (**2020**). doi: 10.1556/1886.2020.00004.
11. Nattramilarasu, Praveen Kumar, Roland Bücker, Fábia Daniela Lobo de Sá, Anja Fromm, Oliver Nagel, In-Fah Maria Lee, Eduard Butkevych, **Soraya Mousavi**, Claudia Genger, Sigri Kløve, Markus M. Heimesaat, Stefan Bereswill, Michal R. Schweiger, Hans Linde Nielsen, Hanno Troeger, and Jörg-Dieter Schulzke. "*Campylobacter concisus* Impairs Sodium Absorption in Colonic Epithelium via ENaC Dysfunction and Claudin-8 Disruption." International Journal of Molecular Sciences 21, no. 2 (**2020**): 373.
12. Heimesaat Markus M., Anna-Maria Schmidt, **Soraya Mousavi**, Ulrike Escher, Nicole Tegtmeyer, Silja Wessler, Gabriele Gadermaier, Peter Briza, Dirk Hofreuter, Stefan

Bereswill, Steffen Backert. “Peptidase PepP is a novel virulence factor of *Campylobacter jejuni* contributing to murine campylobacteriosis.” Gut microbes 12, no. 1 (**2020**): 1770017.

13. Heimesaat, Markus M., Claudia Genger, Sigri Klove, Dennis Weschka, Soraya Mousavi, and Stefan Bereswill. "The Host-Specific Intestinal Microbiota Composition Impacts *Campylobacter coli* Infection in a Clinical Mouse Model of Campylobacteriosis." Pathogens 9, no. 10 (**2020**): 804.
14. Heimesaat, Markus M., Claudia Genger, Nina Biesemeier, Sigri Klove, Dennis Weschka, **Soraya Mousavi**, and Stefan Bereswill. "Inflammatory Immune Responses and Gut Microbiota Changes Following *Campylobacter coli* Infection of IL-10-/-Mice with Chronic Colitis." Pathogens 9, no. 7 (**2020**): 560.
15. Heimesaat, Markus M., **Soraya Mousavi**, Sigri Kløve, Claudia Genger, Dennis Weschka, Andrea Tamas, Dora Reglodi, and Stefan Bereswill. "Pituitary Adenylate Cyclase-Activating Polypeptide Alleviates Intestinal, Extra-Intestinal and Systemic Inflammatory Responses during Acute *Campylobacter jejuni*-induced Enterocolitis in Mice." Pathogens 9, no. 10 (**2020**): 805.
16. Heimesaat, Markus M., Dennis Weschka, Sigri Kløve, Claudia Genger, Nina Biesemeier, **Soraya Mousavi**, and Stefan Bereswill. "Microbiota composition and inflammatory immune responses upon peroral application of the commercial competitive exclusion product Aviguard® to microbiota-depleted wildtype mice." European Journal of Microbiology and Immunology (**2020**).
17. Bandick, Rasmus G., **Soraya Mousavi**, Stefan Bereswill, and Markus M. Heimesaat. "Review of therapeutic options for infections with carbapenem-resistant *Klebsiella pneumoniae*." European Journal of Microbiology and Immunology (**2020**).
18. Hartmann, Minnja S., **Soraya Mousavi**, Stefan Bereswill, Markus M. Heimesaat. “Vitamin E as promising adjunct treatment option in the combat of infectious diseases caused by bacterial including multi-drug resistant pathogens - Results from a comprehensive literature survey.” European Journal of Microbiology and Immunology (**2020**).
19. **Mousavi, Soraya**, Stefan Bereswill, and Markus M. Heimesaat. "Immunomodulatory and antimicrobial effects of vitamin C." European Journal of Microbiology and Immunology 9, no. 3 (**2019**): 73-79.
20. **Mousavi, Soraya**, Fábia Daniela Lobo de Sá, Jörg-Dieter Schulzke, Roland Bücker, Stefan Bereswill, and Markus M. Heimesaat. "Vitamin D in acute campylobacteriosis–results from an intervention study applying a clinical Campylobacter jejuni induced enterocolitis model." Frontiers in immunology 10 (**2019**): 2094.
21. Lobo de Sá, Fábia Daniela, Eduard Butkevych, Praveen Kumar Nattramilarasu, Anja Fromm, **Soraya Mousavi**, Verena Moos, Julia C. Golz, Kerstin Stingl, Sophie Kittler, Diana Seinige, Corinna Kehrenberg, Markus M. Heimesaat, Stefan Bereswill, Jörg-Dieter Schulzke, and Roland Bücker. "Curcumin mitigates immune-induced epithelial barrier dysfunction by *Campylobacter jejuni*." International journal of molecular sciences 20, no. 19 (**2019**): 4830.
22. Schmidt, Anna-Maria, Ulrike Escher, **Soraya Mousavi**, Manja Boehm, Steffen Backert, Stefan Bereswill, and Markus M. Heimesaat. "Protease activity of *Campylobacter jejuni* HtrA modulates distinct intestinal and systemic immune responses in infected secondary abiotic IL-10 deficient mice." Frontiers in cellular and infection microbiology 9 (**2019**): 79.
23. Schmidt, Anna-Maria, Ulrike Escher, **Soraya Mousavi**, Nicole Tegtmeyer, Manja Boehm, Steffen Backert, Stefan Bereswill, and Markus M. Heimesaat. "Immunopathological properties of the *Campylobacter jejuni* flagellins and the adhesin CadF as assessed in a clinical murine infection model." Gut pathogens 11, no. 1 (**2019**): 24.

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