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Dietary factors as modulators of intestinal barrier function and innate
immune signalling: effects of fructose and hop-derived compounds

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Summary

The intestinal barrier is crucial for health and disease. Studies suggest that intestinal barrier dysfunction plays a key role in metabolic diseases such as metabolic dysfunction-associated steatotic liver disease (MASLD) or type 2 diabetes. Increasing evidence indicates that dietary composition critically influences disease onset and progression. A so-called “Western-style” diet, high in saturated fats and sugars like fructose, promotes bacterial translocation and subsequently the risk for developing metabolic diseases. It also increases susceptibility to infections, though mechanisms remain not fully understood. Hop-derived compounds have been shown to possess anti-inflammatory effects and could therefore have beneficial effects on inflammatory diseases, however, their impact in humans at nutritionally relevant doses remains not fully understood. This thesis focused on two main topics: 1) to determine how fructose contributes to the development of MASLD and infections and 2) if an oral supplementation of hop compounds affects immune response of human immune cells. Therefore, the following research questions were addressed in this thesis: 1) Through which mechanisms does fructose alter intestinal barrier function in the setting of MASLD? And are fructose-dependent changes in intestinal nitric oxide homeostasis critical herein? 2) Does dietary fructose intake alter Toll-like receptor 2 (TLR2)-dependent immune responses in humans and if so, which are the underlying mechanisms? 3) Does the acute and long-term intake of hop compounds, like iso- α acids and xanthohumol, modulate lipoteichoic acid- and lipopolysaccharide-induced TLR2 and TLR4 inflammatory responses in human immune cells? Does this affect the course of upper respiratory tract infections?

The results of this dissertation have been published in peer-reviewed journals (Publication 1,2 and 3) or are currently in preparation for publication (Publication 4 and 5).

Research Question 1, Publication 1: Through which mechanisms does fructose alter intestinal barrier function in the setting of MASLD? And are fructose-dependent changes in intestinal nitric oxide homeostasis critical herein?

To investigate the role of intestinal microbiota and barrier dysfunction in MASLD development, male C57BL/6J mice were fed a control diet or a fat- and fructose-rich diet (FFr) with or without antibiotic treatment for seven weeks. Hepatic steatosis development induced by the FFr diet was markedly attenuated by antibiotic treatment, accompanied by undetectable faecal bacteria and TLR4 ligand levels being of the level of controls in the portal blood. In contrast, intestinal barrier dysfunction persisted in FFr-fed mice regardless of antibiotic administration. *Ex vivo* exposure to fructose increased intestinal permeability being related to lower tight junction protein levels irrespective of additional antibiotic treatment, while inhibition of inducible nitric oxide synthase (iNOS) reduced these effects. Overall, the findings indicate that intestinal barrier dysfunction in diet-induced MASLD is primarily driven by fructose-induced disturbances in intestinal nitric oxide (NO) homeostasis rather than by changes in intestinal microbiota.

Research Question 2, Publication 2: Does dietary fructose intake alter Toll-like receptor 2 (TLR2)-dependent immune responses in humans and if so, which are the underlying mechanisms?

The effects of dietary fructose intake on Gram-positive bacterial toxin-induced immune responses were examined in a dietary intervention study combined with *ex vivo* Caco-2 cell culture and primary immune cell experiments. Healthy volunteers consumed a beverage containing either fructose or maltodextrin (glucose control). Monocytes were subsequently isolated and stimulated *ex vivo* with lipoteichoic acid

(LTA), a cell wall component of Gram-positive bacteria. Intake of the fructose-containing beverage significantly enhanced LTA-induced inflammatory responses of monocytes compared with maltodextrin consumption. Furthermore, inhibition of fructose metabolism and blockade of the transcription factor specificity protein 1 (SP1) attenuated fructose-induced TLR2 mRNA expression and cytokine release. Taken together, these findings suggest that fructose-dependent metabolic alterations may be critical in enhancing innate immune responsiveness in humans after fructose consumption.

Research Question 3, Publication 3, 4 and 5: Does the acute and long-term intake of hop compounds, like iso- α acids and xanthohumol, modulate lipoteichoic acid- and lipopolysaccharide-induced TLR2 and TLR4 inflammatory responses in human immune cells? Does this affect the course of upper respiratory tract infections?

To assess whether an iso- α acids (IAA)-rich hop extract affects the lipopolysaccharide (LPS)- and LTA-dependent immune responses, respectively, in healthy humans, monocytes were isolated between 0 and 6 hours after ingestion of different IAA doses by female participants. Cells were stimulated with LPS and LTA, respectively, for 6 hours, identifying 15 mg IAA as the optimal dose. In a subsequent randomized, placebo-controlled crossover study, including male and female volunteers, intake of 15 mg IAA resulted in significantly lower interleukin-6 (IL-6) and interleukin-1 β (IL-1 β) secretion in peripheral blood mononuclear cells (PBMCs) after LTA and LPS stimulation compared to placebo. Mechanistic experiments demonstrated that IAA suppressed c-Jun N-terminal kinase (JNK)-mediated inflammatory signalling without affecting TLR2 activation, while TLR4-dependent signalling pathways were inhibited. These findings support the hypothesis that a single low dose of IAA can modulate inflammatory responses in immune cells of healthy humans (Publication 3 and 4).

As the studies of the own group suggested that xanthohumol may have anti-inflammatory effects on LTA and LPS immune response, acute and long-term human intervention studies were investigated. Acute dose-response experiments in healthy men and women revealed that xanthohumol reduced LPS-induced cytokine release. Based on these findings, a 90-day randomized, placebo-controlled study was conducted in female volunteers receiving 0.75 mg xanthohumol or a placebo twice daily. The long-term intake was related with a significant decrease in symptom burden during cold episodes. Specifically, the duration of symptoms per cold infection and the area under the curve of symptom scores were significantly lower following xanthohumol supplementation in comparison to placebo (Publication 5).

Taken together, results of the first part of this present thesis indicate that intestinal barrier dysfunction and immune response of monocytes are both affected directly by fructose. In a mouse model of fructose- and fat-induced MASLD, hepatic steatosis was associated with increased TLR4 ligand translocation. Antibiotic treatment prevented this effect despite persistent barrier impairment, suggesting that fructose-induced intestinal NO disruption is a key driver of disease development. Human studies further show that dietary fructose enhances innate immune responsiveness through TLR2 signalling. The second part of the thesis suggests that hop-derived compounds such as IAA (15 mg) and xanthohumol (0.75 mg) may dampen TLR2- and TLR4-dependent inflammatory responses in humans, indicating that dietary bioactive compounds found in foods and beverages can modulate immune signalling pathways, even at concentrations found in human nutrition. However, further mechanistic and long-term human intervention studies are required to fully elucidate the underlying pathways and clinical relevance.

Zusammenfassung

Eine intakte Darmbarriere ist entscheidend für Gesundheit und Krankheit. Studien deuten darauf hin, dass eine Funktionsstörung der Darmbarriere eine Schlüsselrolle bei Stoffwechselerkrankungen wie der metabolisch dysfunktionsassoziierte steatotischen Lebererkrankung (MASLD) oder Typ-2-Diabetes spielt. Immer mehr Hinweise deuten darauf hin, dass die Zusammensetzung der Ernährung einen entscheidenden Einfluss auf das Auftreten und Fortschreiten von Krankheiten hat. Eine sogenannte „westliche Ernährung“, die reich an gesättigten Fetten und Zuckern wie Fruktose ist, begünstigt die bakterielle Translokation und damit das Risiko für die Entstehung von Stoffwechselerkrankungen. Sie erhöht auch die Anfälligkeit für Infektionen, wobei die zugrunde liegenden Mechanismen noch nicht vollständig verstanden sind. Aus Hopfen gewonnene Verbindungen zeigen entzündungshemmende Wirkungen und könnten daher positive Auswirkungen auf entzündliche Erkrankungen haben, jedoch ist ihre Wirkung beim Menschen in ernährungsrelevanten Dosen noch nicht vollständig geklärt. Diese Arbeit konzentriert sich auf zwei Hauptthemen, um zu bestimmen, 1) wie Fruktose zur Entwicklung von MASLD und Infektionen beiträgt und 2) ob eine orale Supplementierung mit Hopfenverbindungen die Immunantwort in menschlichen Immunzellen beeinflusst. Daher wurden in dieser Arbeit die folgenden Forschungsfragen behandelt: 1) Über welche Mechanismen verändert Fruktose die Darmbarrierefunktion bei MASLD? Und spielen fruktoseabhängige Veränderungen der Stickstoffmonoxid-Homöostase im Darm dabei eine entscheidende Rolle? 2) Verändert die Aufnahme von Fruktose über die Nahrung die Toll-like Rezeptor 2 (TLR2) -abhängigen Immunreaktionen beim Menschen und wenn ja, welche Mechanismen liegen dem zugrunde? 3) Moduliert die akute und langfristige Aufnahme von Hopfenverbindungen, wie Iso- α Säuren und

Xanthohumol, die durch Lipoteichonsäure und Lipopolysaccharide ausgelösten TLR2- und TLR4- Entzündungsreaktionen in menschlichen Immunzellen? Hat dies Auswirkungen auf den Verlauf von Infektionen der oberen Atemwege?

Die Ergebnisse dieser Dissertation sind entweder in peer-reviewed Fachzeitschriften publiziert (Publikation 1, 2 und 3) oder befinden sich derzeit in Vorbereitung zur Veröffentlichung (Publikation 4 und 5).

Forschungsfrage 1, Publikation 1: Über welche Mechanismen verändert Fruktose die Darmbarrierefunktion bei MASLD? Und spielen fruktoseabhängige Veränderungen der Stickstoffmonoxid-Homöostase im Darm dabei eine entscheidende Rolle?

Um die Rolle der Darmmikrobiota und der Barrierestörung bei der Entwicklung einer MASLD zu untersuchen, wurden männliche C57BL/6J-Mäuse sieben Wochen lang mit einer Kontrolldiät oder einer fett- und fruktosereichen Diät (FFr) mit oder ohne Antibiotikabehandlung gefüttert. Die durch die FFr-Diät induzierte Lebersteatose wurde durch die Antibiotikabehandlung deutlich abgeschwächt, begleitet von nicht nachweisbaren Bakterien und TLR4-Ligandenwerten im portalen Blut die auf dem Niveau der Kontrollgruppe lagen. Im Gegensatz dazu blieb die Darmbarrierefunktionsstörung bei den mit FFr gefütterten Mäusen unabhängig von der Antibiotikagabe bestehen. Die *ex vivo* Exposition gegenüber Fruktose erhöhte die Darmpermeabilität und reduzierte Tight-Junction Proteine unabhängig von Antibiotika, während die Hemmung von induzierbarer Stickstoffmonoxid-Synthase (iNOS) diese Effekte abschwächte. Insgesamt deuten die Ergebnisse darauf hin, dass die Darmbarrierestörung bei der ernährungsbedingten MASLD in erster Linie durch fruktoseinduzierte Störungen der Stickstoffmonoxid (NO)-Homöostase im Darm und weniger durch Veränderungen der Darmmikrobiota verursacht wird.

Forschungsfrage 2, Publikation 2: Verändert die Aufnahme von Fruktose über die Nahrung die Toll-like Rezeptor 2 (TLR2) -abhängigen Immunreaktionen beim Menschen und wenn ja, welche Mechanismen liegen dem zugrunde?

Um die Effekte der Fruktoseaufnahme auf die durch Zellwandbestandteile von Gram-positiven Bakterien induzierte Immunantwort zu untersuchen, wurde eine Ernährungsinterventionsstudie im Crossover-Design durchgeführt, ergänzt durch *ex vivo* Experimente im Caco-2-Zellkulturmodell sowie mit Primärzellen. Gesunde Probanden konsumierten ein Getränk, das entweder Fruktose oder Maltodextrin (Glukosekontrolle) enthielt. Anschließend wurden Monozyten isoliert und *ex vivo* mit Lipoteichonsäure (LTA), ein Bestandteil der Zellwand grampositiver Bakterien, stimuliert. Die Aufnahme des fruktosehaltigen Getränks führte im Vergleich zu Maltodextrin zu einer signifikant verstärkten LTA-induzierten inflammatorischen Antwort der Monozyten. Darüber hinaus schwächten sowohl die Hemmung des Fruktosestoffwechsels als auch die Blockade des Transkriptionsfaktors Specificity Protein 1 (SP1) die fruktoseinduzierte TLR2-mRNA-Expression sowie die Zytokinfreisetzung ab. Zusammenfassend deuten diese Ergebnisse darauf hin, dass fruktoseabhängige metabolische Veränderungen entscheidend für die Verstärkung der angeborenen Immunreaktion beim Menschen nach Fruktosekonsum sein könnten.

Forschungsfrage 3, Publikation 3, 4 und 5: Moduliert die akute und langfristige Aufnahme von Hopfenverbindungen, wie Iso- α Säuren und Xanthohumol, die durch Lipoteichonsäure und Lipopolysaccharide ausgelösten TLR2- und TLR4- Entzündungsreaktionen in menschlichen Immunzellen? Hat dies Auswirkungen auf den Verlauf von Infektionen der oberen Atemwege?

Um zu beurteilen, ob ein Iso- α Säuren (IAA)-reicher Hopfenextrakt die Lipopolysaccharid- (LPS)- und LTA-abhängige Immunantwort bei gesunden Menschen beeinflusst, wurden Monozyten zwischen 0 und 6 Stunden nach der Einnahme unterschiedlicher IAA-Dosen von weiblichen Probanden isoliert. Die Zellen wurden jeweils 6 Stunden lang mit LPS oder LTA stimuliert, wobei 15 mg IAA als optimale

Dosis ermittelt wurde. Anschließend führte die Einnahme von 15 mg IAA in einer randomisierten, placebokontrollierten Crossover-Studie mit männlichen und weiblichen Probanden zu einer signifikant geringeren Interleukin-6- (IL-6)- und Interleukin-1 β - (IL-1 β)-Sekretion in peripheren mononukleären Blutzellen (PBMCs) nach einer LTA- oder LPS-Stimulation im Vergleich zu Placebo. Ergänzende *in vitro* Experimente zeigten, dass IAA die durch c-Jun N-terminale Kinase (JNK) vermittelte Entzündungssignalübertragung unterdrückte, ohne die TLR2-Aktivierung zu beeinflussen, während der TLR4-abhängige Signalweg gehemmt wurde. Diese Ergebnisse stützen die Hypothese, dass eine einzelne niedrige Dosis IAA die Entzündungsreaktionen in den Immunzellen gesunder Menschen modulieren kann (Publikation 3 und 4).

Da die Studien der eigenen Gruppe darauf hindeuten, dass Xanthohumol entzündungshemmende Wirkungen auf die Immunantwort nach LTA bzw. LPS-Stimulation hat, wurden diese Effekte in akuten und langfristigen Humaninterventionsstudien untersucht. Akute Dosis-Wirkungs-Experimente bei gesunden Frauen und Männern zeigten, dass Xanthohumol die LPS-induzierte Zytokinfreisetzung reduzierte. Auf der Grundlage dieser Ergebnisse wurde eine 90-tägige randomisierte, placebokontrollierte Studie mit weiblichen Probanden durchgeführt, welche zweimal täglich 0,75 mg Xanthohumol oder ein Placebo erhielten. Die langfristige Einnahme ging mit einer signifikanten Verringerung der Symptombelastung während Erkältungsphasen einher. Konkret waren die Symptombdauer pro Erkältungsinfektion und die Fläche unter der Kurve der Symptomschwere nach einer Xanthohumol-Supplementierung im Vergleich zur Placebo-Gruppe signifikant geringer (Publikation 5).

Insgesamt deuten die Ergebnisse des ersten Teils dieser Arbeit darauf hin, dass sowohl die Funktionsstörung der Darmbarriere als auch die Immunantwort der Monozyten direkt durch Fruktose beeinflusst werden. In einem Mausmodell für fruktose- und fettinduzierte MASLD war die Lebersteatose mit einer erhöhten Translokation von TLR4-Liganden verbunden. Eine Antibiotikabehandlung verhinderte diesen Effekt trotz anhaltender Beeinträchtigung der Barriere, was darauf hindeutet, dass die durch Fruktose induzierte Störung des intestinalen NO-Haushalts ein wesentlicher Treiber der Krankheitsentwicklung ist. Studien am Menschen zeigen zudem, dass Fruktose aus der Nahrung die Reaktionsfähigkeit des angeborenen Immunsystems über die TLR2-Signalübertragung verstärkt. Der zweite Teil der Arbeit legt nahe, dass aus Hopfen gewonnene Verbindungen wie IAA (15 mg) und Xanthohumol (0,75 mg) TLR2- und TLR4-abhängige Entzündungsreaktionen beim Menschen dämpfen können. Dies deutet darauf hin, dass bioaktive Verbindungen in Lebensmitteln und Getränken die Immunsignalwege modulieren können, selbst in Konzentrationen, wie sie in der menschlichen Ernährung vorkommen. Es sind jedoch weitere mechanistische und langfristige Interventionsstudien am Menschen erforderlich, um die zugrunde liegenden Signalwege und die klinische Relevanz vollständig aufzuklären.

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Abbreviations

AMR	Antimicrobial resistance
CD14	Cluster of differentiation 14
FFr	Fat- and fructose-rich diet
HEK	Human embryonic kidney
IAA	Iso- α acids
IL-1 β	Interleukin-1 β
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
IRAK1	Interleukin 1 receptor associated kinase 1
IRAK4	Interleukin 1 receptor associated kinase 4
JNK	C-Jun N-terminal kinase
LBP	LPS-binding protein
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MASLD	Metabolic dysfunction-associated steatotic liver disease
MD-2	Myeloid differentiation protein 2
MyD88	Myeloid differentiation factor 88
NCDs	Non-communicable diseases
Nf κ B	Nuclear factor kappa B
NK	Natural killer
NO	Nitric oxide
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PRRs	Pattern recognition receptors
SP1	Specificity protein 1
TIRAP	Toll/interleukin-1 receptor domain containing adaptor protein
TLR	Toll-like receptor
TNF- α	Tumor necrosis factor- α
TRAF6	Tumor necrosis factor receptor associated factor 6
URTI	Upper respiratory tract infection

1. Introduction

1.1. The gut-liver axis and innate immune activation

1.1.1. The intestinal barrier as a regulator of gut-liver communication

The term gut-liver axis refers to the close interaction between the intestine and the liver, which also involves a complex interplay between the gut barrier, the gut microbiome and the immune system¹. The intestinal barrier acts here as a selective filter that allows the uptake of nutrients while simultaneously preventing toxic environmental substances, pathogens and pathogen-associated molecular patterns (PAMPs) from entering the systemic circulation². Structurally, the intestinal barrier consists of three major components: the mucus layer, a single layer of epithelial cells, and the underlying lamina propria containing various immune cells (see Figure 1)^{3,4}. The mucus layer comprises an outer microbiota-colonized layer and an inner sterile layer⁴. Specialized epithelial cells include mucus-producing goblet cells, antimicrobial peptide-secreting Paneth cells, enteroendocrine cells regulating gut physiology, intestinal stem cells responsible for epithelial renewal, as well as dendritic cells and M cells involved in antigen sampling and transcytosis⁴. The mucus layer limits direct contact between the epithelium and luminal microorganisms and pathogens, while epithelial tight junctions regulate paracellular permeability⁴ and represent critical, dynamically regulated determinants of intestinal barrier function⁵. Despite highly specialized epithelial and vascular barriers that tightly regulate transport across the intestinal mucosa, microbial products, antigens and even bacteria can translocate into the portal circulation⁶⁻⁸. In this context, excessive nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) during inflammation, directly impairs intestinal barrier integrity by disrupting tight junctions and promoting epithelial cell apoptosis, thereby facilitating PAMPs translocation⁹.

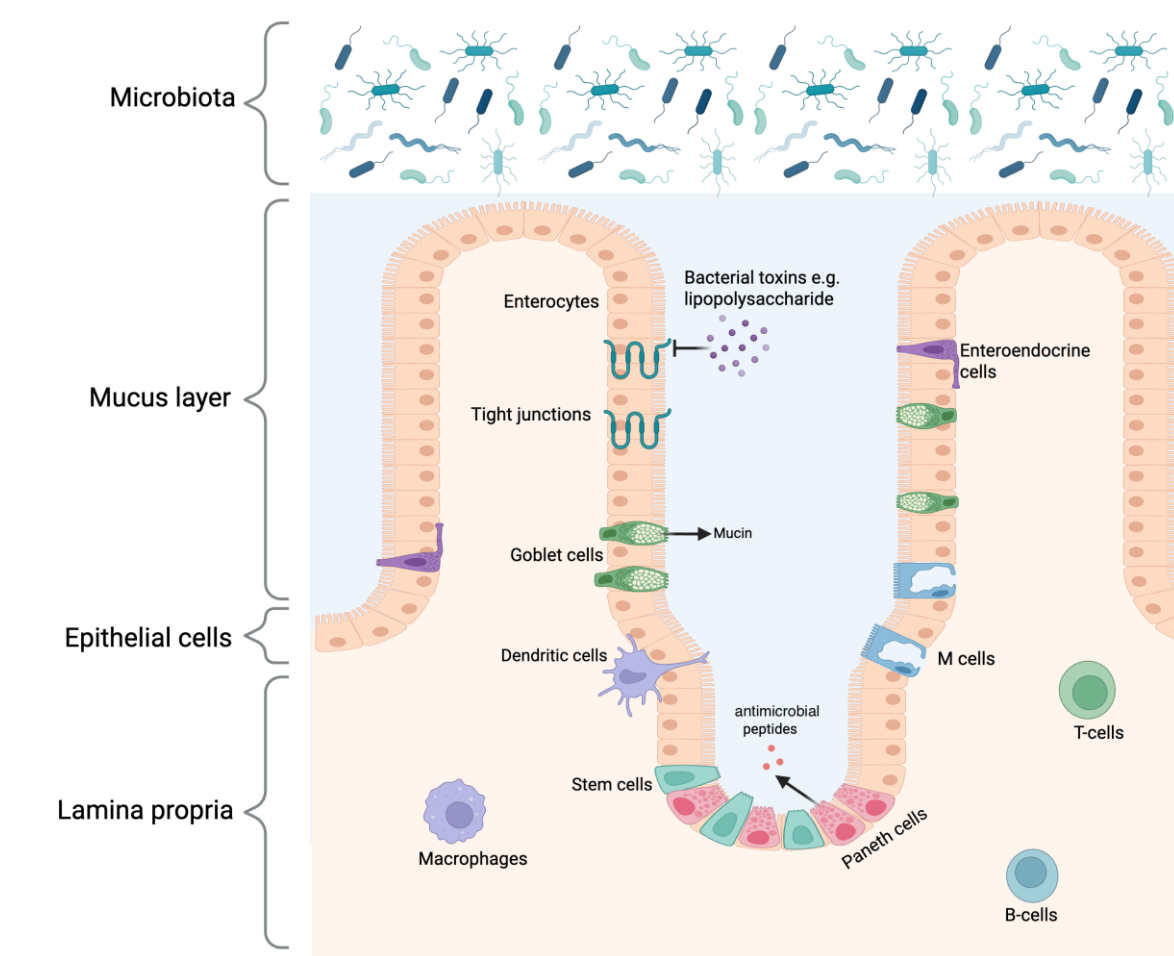


Figure 1: Schematic illustration of an intact intestinal barrier

The intestinal barrier consists of microbiota, the mucus layer, a single layer of epithelial cells and the lamina propria. The lamina propria contains various cells of the innate and adaptive immune system, including macrophages as well as T- and B-cells. The epithelial layer is composed of absorptive enterocytes as well as several specialised cell types, such as mucus-producing goblet cells, enteroendocrine cells, stem cells as well as Paneth cells. Tight junctions with selectively permeable transport proteins within the epithelial layer regulate paracellular permeability. The mucus layer is further supported and complemented by the intestinal microbiota. Modified from Berghheim and Moreno-Navarrete⁵ and Di Sabatino et al.⁴. Figure created with BioRender.

The portal vein thereby represents the central connection between the intestine and the liver, transporting nutrients as well as microbial-derived products directly to the liver^{10,11}. Furthermore, some evidence suggests that the disruption of the gut-liver axis (e.g. through an unhealthy diet or overweight) alters the balance between immune activation and tolerance, and the resulting immune deficiency contributes significantly to the pathogenesis and progression of liver diseases, particularly metabolic dysfunction-associated steatotic liver disease (MASLD)¹².

1.1.2. Innate immune activation by pathogen-associated molecular patterns

Disruption of the intestinal barrier allows microbial components, including lipopolysaccharide (LPS) from Gram-negative bacteria and lipoteichoic acid (LTA) from Gram-positive bacteria, collectively referred to as PAMPs, to translocate via the portal vein into the systemic circulation, where they can trigger pro-inflammatory signalling pathways (see Figure 2)¹³.

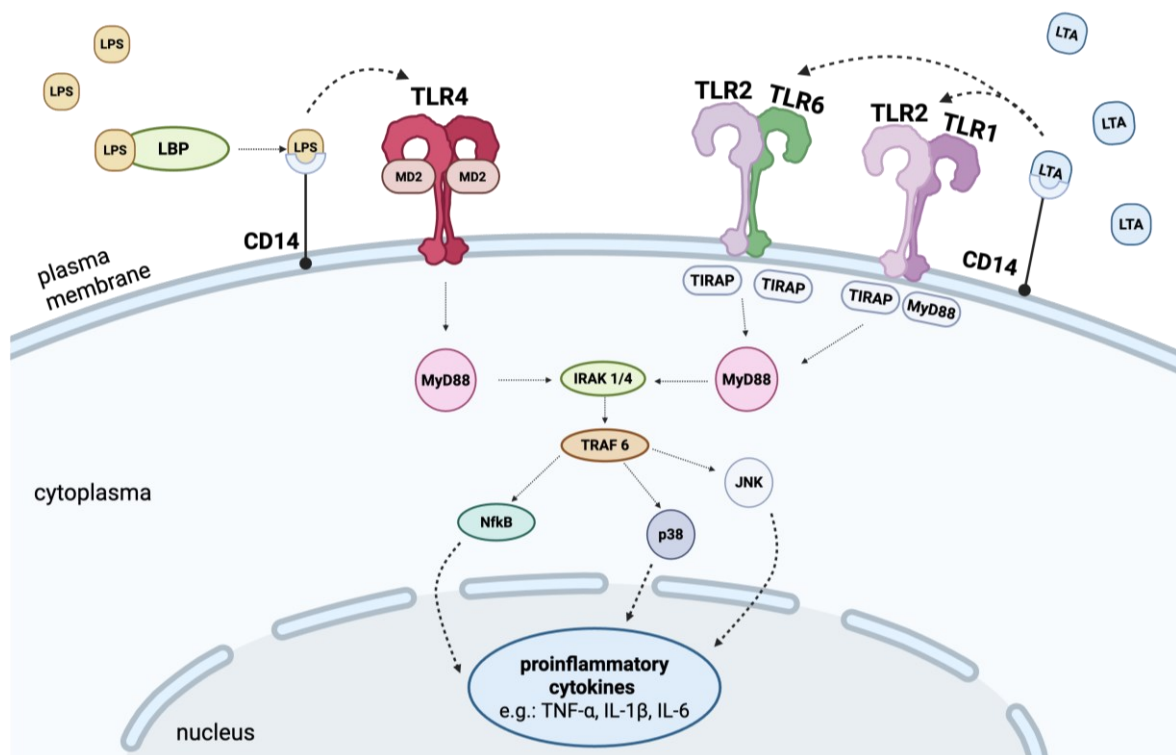


Figure 2: Schematic illustration of Toll-like receptor 2 and 4 signalling pathways

LPS binds to LBP and is transferred to CD14, facilitating its interaction with TLR4. This causes the release of pro-inflammatory cytokines via MyD88. After binding to CD14, LTA can be recognized by TLR2 which is mostly present as a heterodimer (with TLR1 or TLR6). Recognition of TIRAP associated with TLR2 leading to the recruitment of MyD88 and activation of the downstream signalling pathway. CD14: Cluster of differentiation 14; IL: Interleukin; IRAK1: Interleukin 1 receptor associated kinase 1; IRAK4: Interleukin 1 receptor associated kinase 4; JNK: C-Jun N-terminal kinase; LBP: LPS-binding protein; LPS: Lipopolysaccharide; LTA: Lipoteichoic acid; MD-2: Myeloid differentiation protein 2; MyD88: Myeloid differentiation factor 88; NfκB: Nuclear factor kappa B; TIRAP: Toll/interleukin-1 receptor domain containing adaptor protein; TLR: Toll-like receptor; TNF: Tumor necrosis factor; TRAF6: Tumor necrosis factor receptor associated factor 6. Modified from Chen et al.¹⁴ and Mansouri et al.¹⁵. Figure created with BioRender.

PAMPs can be recognized through Pattern recognition receptors (PRRs) which play a central role in innate immune responses and are expressed by various immune and non-immune cell types, including macrophages, neutrophils, dendritic cells, fibroblasts and epithelial cells^{16,17}. Among PRRs, Toll-like receptor 4 (TLR4) is the most extensively studied member of the TLR family and is primarily activated by LPS, a structural component of the outer cell membrane of Gram-negative bacteria such as *Enterobacteriaceae*¹⁸. Studies in rodents and humans indicate that the activation of TLRs and particularly TLR4, plays a critical role in the onset and progression of MASLD^{19,20}. LPS is released during bacterial lysis and consists of three structural domains: the O antigen, the core polysaccharide and lipid A, which represents the biologically active endotoxin²¹. While still under debate, studies suggest that LPS can be recognized by TLR4 after binding to LPS-binding protein (LBP) and subsequent transfer to the co-receptor cluster of differentiation 14 (CD14)²². This complex facilitates the interaction of LPS with the TLR4/MD-2 heterodimer, thereby initiating intracellular signalling cascades^{23,24}.

Downstream signalling involves recruitment of the adaptor protein myeloid differentiation factor 88 (MyD88), leading to activation of interleukin 1 receptor-associated kinase 4 (IRAK4)²⁵. IRAK4 subsequently phosphorylates interleukin 1 receptor associated kinase 1 (IRAK1) and leads to the formation of the MyD88-IRAK4-IRAK1 complex^{14,15,26}. Subsequently, tumor necrosis factor receptor associated factor 6 (TRAF6) is activated, which results in the translocation of the transcription factor nuclear factor kappa B (NfκB) to the nucleus^{27,28}. Activation of this pathway stimulates the innate immune response and induces the expression of pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6)²⁹. In parallel, TRAF6-dependent activation of p38 and c-Jun N-

terminal kinase (JNK) pathways further contributes to inflammatory cytokine production^{30,31}.

In addition to TLR4, TLR2 plays a key role in the recognition of Gram-positive bacterial components like LTA^{32,33}. Following binding to CD14, LTA is recognized by TLR2, resulting in recruitment of the adaptor protein Toll/interleukin-1 receptor domain containing adaptor protein (TIRAP) leading to the recruitment of MyD88³⁴. This initiates downstream signalling pathways similar to those activated by TLR4 (see Figure 2)³⁵.

Recognition of PAMPs through TLRs activates innate immune signalling pathways, resulting in the release of pro-inflammatory cytokines such as IL-6 and IL-1 β , along with other inflammatory mediators³⁶. These early immune reactions are part of the innate immune system, which serves as the first line of defense against pathogens³⁷. The human immune system is divided into the innate (non-specific) and adaptive (specific) immune system³⁷. The innate defense includes the physical barriers of the skin and mucous membranes, as well as immune cells and the release of soluble mediators such as cytokines and acute-phase proteins³⁸. After pathogens crossing the physical barrier, innate immune responses are rapidly initiated. Pathogens may be eliminated through phagocytosis or processed and presented as antigens, thereby triggering activation of the adaptive immune system³⁸. Leukocytes represent the cellular basis of immune defense and include granulocytes and peripheral blood mononuclear cells (PBMCs)³⁷. The latter comprising lymphocytes, monocytes and dendritic cells^{37,39}. Within this compartment, lymphocytes mediate humoral and cell-mediated immune response, while monocytes and dendritic cells function as key sensors of microbial-derived signals. Monocytes circulate as precursors of tissue macrophages and contribute to host defense through phagocytosis

and cytokine production⁴⁰⁻⁴². The regulation of cytokine production by monocytes is a key factor in determining the severity of symptoms, the duration of the illness and the overall burden of disease in upper respiratory tract infections^{43,44}

1.2. Diet, intestinal barrier integrity and bacterial infections

1.2.1. Bacterial infections and innate immune responses

Despite the widespread availability of antibiotic treatments, even in poorer regions, bacterial infections are still among the leading causes of death worldwide and constitute a major global health concern⁴⁵. In 2019, approximately 7.7 million deaths were associated with 33 bacterial pathogens, making them the second leading cause of death globally⁴⁶. Five pathogens accounted for more than 500,000 deaths, including the Gram-positive bacterium *Staphylococcus aureus*, which caused the highest number of deaths in people older than 15 years⁴⁶. Another major pathogen is the Gram-negative bacterium *Escherichia coli*, which represents a leading cause of bloodstream, urinary tract and intra-abdominal infections worldwide⁴⁷. Over the past 30 years, resistance to Gram-negative bacterial infections has increased dramatically⁴⁵. Globally, it is estimated that by 2050, 1.9 million deaths will be attributed to antimicrobial resistance (AMR) (see Figure 3)⁴⁵.

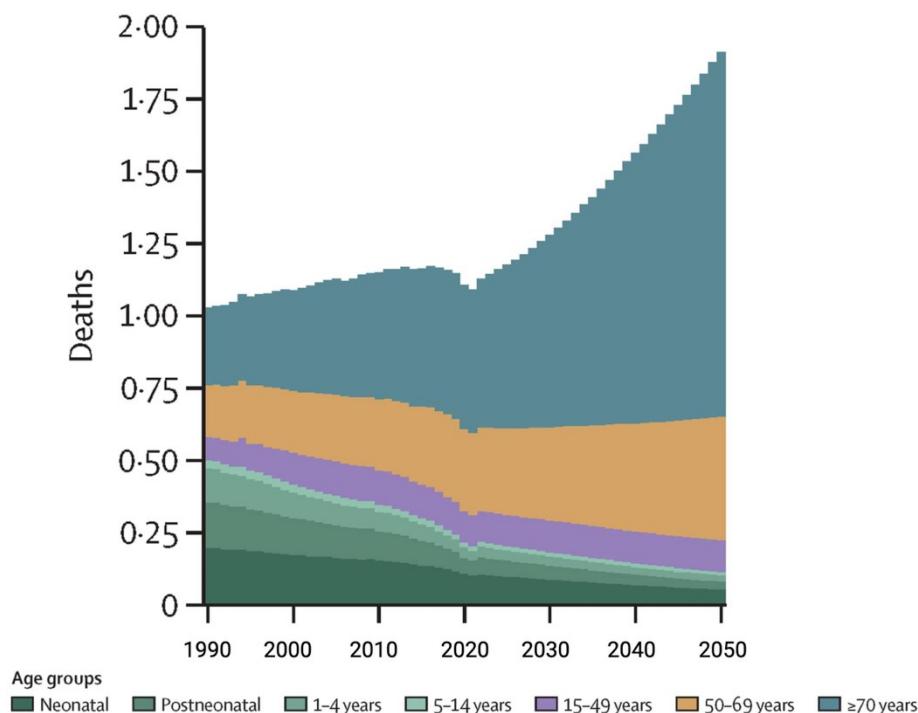


Figure 3: Deaths attributable to antimicrobial resistance by age groups, 1990-2050

The overall forecasted death numbers attributable to AMR show opposite trends by age. AMR deaths in children younger than 5 years are decreasing, deaths of people 70 years and older will increase. AMR: antimicrobial resistance. Modified from GBD Antimicrobial Resistance Collaborators⁴⁵. Figure created with BioRender.

Bacterial virulence factors, including cell wall components of Gram-negative (e.g. LPS) and Gram-positive bacteria (e.g. LTA), critically contribute to infection severity by inducing inflammatory responses and immune dysregulation⁴⁸⁻⁵⁰. In the context of increasing AMR, understanding host immune responses to bacterial virulence factors has become increasingly important for the development of alternative therapeutic strategies, particularly as the burden of AMR-related deaths is expected to grow in the elderly population⁵¹.

1.2.2. Fructose-induced intestinal barrier dysfunction and immune dysregulation

In recent years, alterations of the intestinal barrier as well as changes in gut microbiota composition have increasingly been discussed as key modulators of human

health^{52,53}. Studies have shown that diet composition is a major modulating factor of the intestinal barrier and microbiota^{54,55}. In particular, a diet characterized by energy-dense, nutrient-poor and highly processed foods and beverages and therefore rich in saturated fats and sugars and low in fiber, is considered a risk factor for the development of non-communicable diseases (NCDs) such as overweight and obesity⁵⁶, type 2 diabetes^{57,58}, high blood pressure⁵⁹ and cardiovascular disease^{60,61}, as well as cancer^{62,63} and MASLD⁶⁴. This dietary pattern is often referred to as the “Western-style” diet and is also discussed as one of the key modulators of the intestinal barrier, microbiota composition and disease development⁶⁵.

“Western-style” diets are typically characterized by high intakes of processed foods, saturated fats, and added sugars, particularly through sugar-sweetened beverages, processed foods and sweets^{66,67}. Excessive sugar consumption has been linked to an increased incidence of NCDs including type 2 diabetes⁶⁸, cardiovascular disease⁶⁹ and cancer^{70,71}. The increasing global prevalence of NCDs linked to excessive sugar consumption constitutes a major public health challenge, accounting for approximately 35 million deaths each year⁷². Studies further show that chronic consumption of a “Western-style” diet not only increases the risk for NCDs, but also the susceptibility for infectious diseases by inducing low-grade inflammation and impairing effective immune response^{73,74}.

In the following, present knowledge on the effects of sugar and especially fructose on intestinal barrier is summarized. Experimental studies in rodents demonstrate that dietary sugars not only modulate gut microbial composition but also impair intestinal barrier function by disrupting tight junction integrity^{20,75}. Notably, fructose appears to exert more pronounced detrimental effects on the intestinal barrier compared to other monosaccharides⁷⁶⁻⁷⁸. Bergheim et al. showed that fructose-induced

barrier dysfunction was absent when fructose was replaced by glucose, indicating sugar-specific effects on intestinal permeability⁷⁶. These differences may be explained by the distinct intestinal uptake and insulin-independent metabolism of fructose in enterocytes, which bypasses key regulatory steps of glycolysis and thereby alters intracellular signalling compared to glucose^{79,80}.

Although the underlying mechanisms are not yet fully understood, increasing evidence suggests that alterations in intestinal NO homeostasis may contribute to fructose-induced barrier dysfunction^{81,82}. NO is a key signalling molecule in the gastrointestinal tract, where it plays a dual role in maintaining mucosal homeostasis but can also promote tissue damage when produced in excess⁸³. Its production is tightly regulated by the availability of the substrate L-arginine, which is metabolized by both nitric oxide synthases (NOS) and arginases⁸⁴. While NOS converts L-arginine to NO, arginase competes for the same substrate and thereby limits NO production⁸⁵. Consequently, an imbalance between these pathways can lead to dysregulated NO levels and impaired epithelial function⁸⁵.

Fructose-rich diets have been shown to alter intestinal permeability and NO-homeostasis⁸⁶. Previous studies from our group demonstrated that fructose intake increases intestinal NO production while simultaneously reducing arginase activity, resulting in an imbalance in NO regulation^{86,87}. These studies further suggest that elevated NO levels are associated with structural alterations of tight junction proteins and increased epithelial permeability, thereby contributing to intestinal barrier dysfunction^{86,87}.

In industrialized countries, metabolic diseases such as MASLD and type 2 diabetes are associated not only with metabolic complications but have also been proposed with increased susceptibility to infections, a more severe disease course and higher

infection-related mortality compared to metabolically healthy individuals^{88,89}. Fructose metabolism additionally promotes uric acid production and oxidative stress, which may further exacerbate metabolic and inflammatory disturbances (for overview see⁸²). Chronic low-grade inflammation has been proposed to impair liver integrity and immune function, thereby contributing to immune dysregulation⁸⁹. However, the underlying mechanisms linking metabolic disease and altered immune responses remain incompletely understood. In this context, diet-induced intestinal barrier dysfunction and subsequent bacterial translocation may represent a critical link. Together, these findings indicate that fructose acts as a potent dietary modulator of intestinal barrier integrity, innate immune activation and bacterial translocation, thereby influencing host susceptibility to bacterial infections.

1.3. Hop compounds as modulator of inflammation and infection in human health

Nutritional compounds play an important role in the modulation of inflammation and immune responses, contributing to the prevention and management of infectious and inflammatory diseases. A wide range of nutritional compounds has been shown to affect immune pathways through immunomodulatory and antimicrobial mechanisms⁹⁰. Despite promising evidence, their clinical application remains complex and requires further investigation.

Hop (*humulus lupulus*) and hop-derived compounds have been used in humans for centuries, both in brewing and traditional medicine, for the treatment of various disorders, including inflammation, infections, sleep disturbances and gastrointestinal complaints (e.g. mild abdominal pain, bloating or nausea)⁹¹. Traditionally, hop cones were applied as mild sedatives and bitter agents to stimulate gastric function, effects that have largely been attributed to hop resins⁹². In recent years, hop-derived

compounds have attracted increasing scientific interest due to their potential immunomodulatory, anti-inflammatory and antimicrobial properties⁹³.

The major bioactive constituents of ripe hop cones include bitter acids, terpenes and prenylated flavonoids, particularly the chalcone xanthohumol^{94,95}. Bitter acids are classified into α -acids and β -acids, with β -acids containing an additional prenyl group. The concentrations of β -acids, along with xanthohumol, increase during maturation, highlighting their biological relevance for the plant⁹⁶⁻⁹⁸.

1.3.1. Iso- α acids in inflammation and infection

The antibacterial properties of hop-derived compounds have mainly been attributed to iso- α acids (IAA) and the prenylated flavonoid xanthohumol⁹⁹⁻¹⁰¹. IAA are hydrophobic derivatives of α -acids formed during the isomerization of humulone (see Figure 4)¹⁰². Their antimicrobial activity has been related to hydrophobic interactions with bacterial cell walls and membranes, resulting in membrane disruption and enhanced antibacterial efficacy^{99,100}.

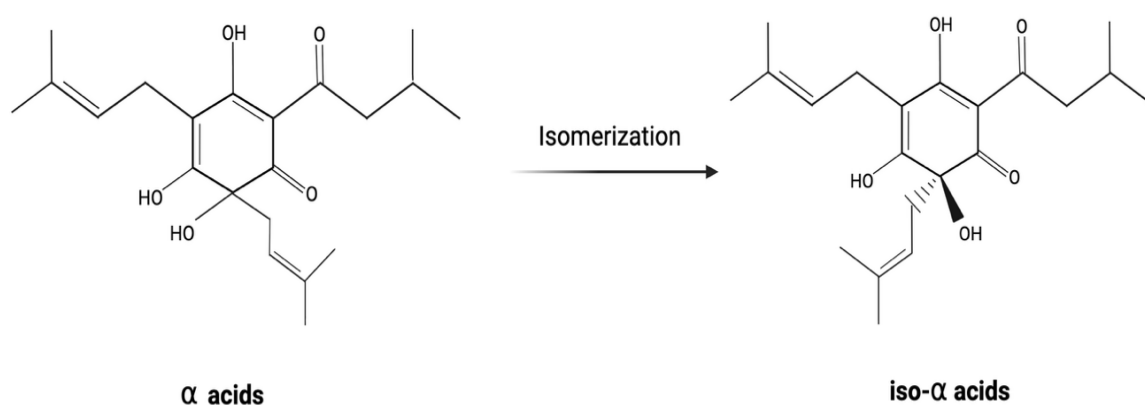


Figure 4: Isomerization of α acids found in hop to iso- α acids

Modified from Laurent et al.¹⁰³. Figure created with BioRender.

IAA are rapidly absorbed in humans in a concentration-dependent manner and have been shown to partially reduce hepatic lipid accumulation and inflammatory processes¹⁰⁴. Human intervention studies have demonstrated that, following ingestion of IAA through beer consumption, plasma concentrations increase rapidly, reach peak levels approximately 30-60 minutes after intake and decline within approximately six hours, although their metabolic pathways remain incompletely characterized^{105,106}. Despite these promising findings, it remains unclear whether the amounts of IAA achievable through nutritional doses in commercially available products, including non-alcoholic beers, are sufficient to achieve clinically relevant immunomodulatory or anti-inflammatory effects. Most studies demonstrating antimicrobial effects used relatively high concentrations or were conducted in rodent models^{104,107,108}.

1.3.2. Xanthohumol in inflammation and infection

Xanthohumol, the most abundant prenylated chalcone in hops, represents another major bioactive hop compound. It is localized in the lupulin glands of hop cones and contributes approximately 0.1–1% to the dry weight of hop cones^{109,110}.

During wort boiling xanthohumol is largely isomerized to isoxanthohumol (see Figure 5), and consequently, beer typically contains up to approximately 3 mg/l of isoxanthohumol and around 1 mg/l of xanthohumol, depending primarily on the amount and composition of hops used^{95,111}. Xanthohumol is rapidly absorbed in a concentration-dependent manner, with peak plasma levels occurring approximately one hour after ingestion^{112,113}.

Studies suggest that xanthohumol exhibits a broad range of pharmacological activities. *In vitro* and *in vivo* studies have demonstrated effects against Gram-positive

bacteria, including *Staphylococcus aureus* and *Streptococcus mutans*^{114,115}. Moreover, antiviral¹¹⁶, antifungal¹¹⁷ and antiparasitic¹¹⁸ activities have been reported. In addition, *in vitro* studies have shown that xanthohumol suppresses the expression of iNOS, NO, interferon- γ and other pro-inflammatory mediators in macrophages^{119,120}, as well as exerting antifibrotic effects in hepatic stellate cells¹²¹. However, many of these effects have been observed at pharmacological doses substantially higher than those achievable through dietary intake. Notably, Jung et al. demonstrated that even low, nutritionally relevant doses of xanthohumol, derived from a xanthohumol-rich hop extract, can attenuate inflammatory responses in human immune cells as early as one hour after ingestion^{122,123}. However, evidence regarding long-term effects and the impact on disease development in humans is still lacking.

Notably, both IAA and xanthohumol exhibit favorable safety profiles in preclinical studies^{107,124,125}. Nevertheless, further research is required to determine whether nutritionally relevant doses, such as those obtained through moderate beer consumption, are sufficient to induce clinically meaningful anti-infective or immunomodulatory effects in humans and to clarify potential interindividual differences in response.

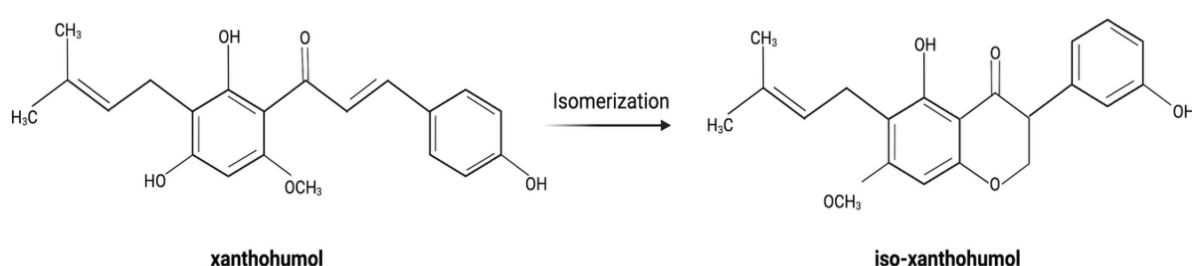


Figure 5: Isomerization of xanthohumol found in hop to iso-xanthohumol
 Modified from Magalhaes et al.¹²⁶. Figure created with BioRender.

1.4. Research question and objective of the dissertation

The aim of this dissertation was to investigate nutrition-dependent effects of fructose on intestinal barrier function, innate immune responses, and the development of MASLD, as well as the potential immunomodulatory effects of the hop-derived compounds IAA and xanthohumol at nutritionally relevant concentrations. The following research questions were addressed:

1. Through which mechanisms does fructose alter intestinal barrier function in the setting of MASLD? And are fructose-dependent changes in intestinal nitric oxide homeostasis critical herein?
2. Does dietary fructose intake alter Toll-like receptor 2 (TLR2)-dependent immune responses in humans and if so, which are the underlying mechanisms?
3. Does the acute and long-term intake of hop compounds, like iso- α acids and xanthohumol, modulate lipoteichoic acid- and lipopolysaccharide-induced TLR2 and TLR4 inflammatory responses in human immune cells? Does this affect the course of upper respiratory tract infections?

2. Publications

Publication 1, 2 and 3 are Open Access articles already been published. Publication 4 and 5 currently being processed for publication.

2.1. Publication 1: Antibiotics attenuate diet-induced nonalcoholic fatty liver disease without altering intestinal barrier dysfunction

Annette Brandt*, [Katja Csarmann*](#), Angélica Hernández-Arriaga, Anja Baumann, Raphaela Staltner, Emina Halilbasic, Michael Trauner, Amélia Camarinha-Silva, Ina Bergheim. *J Nutr Biochem.* 2024 Jan; 123:109495. doi: 10.1016/j.jnutbio.2023.109495

*Authors contributed equally

Highlights:

- In fat and fructose-fed mice, administration of antibiotics prevented the development of hepatic steatosis and early signs of inflammation.
- Although antibiotics eliminate bacterial load in the intestine and normalise TLR4 ligands in portal vein blood, increased intestinal permeability and iNOS expression remain unchanged.
- Everted gut sacs from naïve mice showed increased permeability and loss of tight junction proteins upon exposure to fructose, independent of antibiotic treatment.
- Inhibition of NO synthesis (aminoguanidine) protected against fructose-induced barrier disruption, indicating that disturbances in intestinal NO homeostasis are an important driver.

Contribution to the manuscript:

- Laboratory analyses of tissue and everted gut sac samples
- Analysis of data and statistical evaluation
- Writing – review and editing

Total contribution: 20%

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RESEARCH PAPER

Antibiotics attenuate diet-induced nonalcoholic fatty liver disease without altering intestinal barrier dysfunction

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Abstract

To date the role of the alterations of intestinal microbiota in the development of intestinal barrier dysfunction in settings of nonalcoholic fatty liver disease (NAFLD) has not been fully understood. Here, we assessed the effect of antibiotics on development of NAFLD and their impact on intestinal barrier dysfunction. Male C57BL/6J mice were either pair-fed a liquid control diet (C) or fat- and fructose-rich diet (FFr) +/- antibiotics (AB, ampicillin/vancomycin/metronidazole/gentamycin) for 7 weeks. Fasting blood glucose was determined and markers of liver damage, inflammation, intestinal barrier function, and microbiota composition were assessed. The development of hepatic steatosis with early signs of inflammation found in FFr-fed mice was significantly abolished in FFr+AB-fed mice. Also, while prevalence of bacteria in feces was not detectable and TLR4 ligand levels in portal plasma were at the level of controls in FFr+AB-fed mice, impairments of intestinal barrier function like an increased permeation of xylose and iNOS protein levels persisted to a similar extent in both FFr-fed groups irrespective of AB use. Exposure of everted small intestinal tissue sacs of naïve mice to fructose resulted in a significant increase in tissue permeability and loss of tight junction proteins, being not affected by the presence of AB, whereas the concomitant treatment of tissue sacs with the NOS inhibitor aminoguanidine attenuated these alterations. Taken together, our data suggest that intestinal barrier dysfunction in diet-induced NAFLD in mice may not be predominantly dependent on changes in intestinal microbiota but rather that fructose-induced alterations of intestinal NO-homeostasis might be critically involved.

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This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>)**Keywords:** Antibiotic; Intestinal barrier function; Fatty liver; Tight junction; Endotoxin; Nitric oxide.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is by now the most frequently diagnosed liver disease in the world affecting ~30% of the general world population [1]. Results of a recent study further suggest that liver cirrhosis resulting from nonalcoholic steatohepatitis (NASH), the progressive variant of NAFLD, is the leading cause of liver transplantation in the U.S. [2]. However, despite intense efforts and marked progress in the understanding of molec-

ular changes and mechanisms associated with the development of NAFLD, life-style interventions with a specific focus on restrictions of caloric intake and an increase of physical activity are still the key recommendation for patients with NAFLD.

A genetic predisposition, low physical activity, and general over-nutrition being rich in saturated fats and sugars like fructose are among the key risk factors for the development of NAFLD (for overview see [3]). Furthermore, alterations at the level of the gut including changes in microbiota and barrier function, may also

Abbreviations: AB, antibiotics; ALT, alanine aminotransferase; C, control diet; Fr, fructose; FFr, fat- and fructose-rich diet; IL, interleukin; iNOS, inducible nitric oxide synthase; KRH buffer, 1 x Krebs-Henseleit-bicarbonate buffer supplemented with 0.2% bovine serum albumin; MCD, methionine choline deficient diet; NO, nitric oxide; NAFLD, nonalcoholic fatty liver disease; NAS, NAFLD Activity Score; NASH, nonalcoholic steatohepatitis; nMDS, nonmetric multidimensional scaling; PCR, polymerase chain reaction; SEAP, secreted embryonic alkaline phosphatase; SEM, standard error of the means; TBARS, thiobarbituric acid reactive substances; TLR4, toll-like receptor 4; ZO-1, zonula occludens-1.

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contribute to the development of the disease [4–8]. In line with these findings, it has been shown that even the early stages of the disease are associated with increased blood levels of bacterial endotoxin and increased expression levels of toll-like receptors (TLR) in liver and peripheral blood mononuclear cells [4,9–11]. Also, it has been shown that tight junction protein levels in small intestinal tissue are lower in patients with NAFLD and animals with diet-induced NAFLD [6,7,12,13]. Furthermore, the development of steatohepatitis and fibrosis has also been related to the prevalence of specific *Bacteroides* and *Ruminococcus* in adults while in children *Prevotella copri* was associated with more severe fibrosis [5,14]. Moreover, treatment with antibiotics has been shown to prevent the development of NAFLD in rodents when a mix of antibiotics targeting both Gram positive and Gram negative bacteria was employed [10,15–17]. Contrasting these findings, studies using (sub-)therapeutic doses of different antibiotics inflicting a decrease in microbial diversity and relative abundance of specific species have been reported to exacerbate the effects of the respective diet on the liver in animal models [18,19]. Others have reported that the treatment of both humans and mice with rifaximin may have beneficial effects in settings of NAFLD if being associated with a shift in bacterial composition [20,21]. However, if the protective effects of the loss/diminishment or shift of intestinal microbiota affect intestinal barrier function in small intestine in settings of diet-induced early NAFLD has not yet been clarified. Starting from this background we assessed the effect of an antibiotic mixture (ampicillin, vancomycin, metronidazole, gentamycin) on the development of diet-induced NAFLD and intestinal barrier function in mice.

2. Material and methods

2.1. Animals and treatment

Male C57BL/6J mice (6 weeks old upon arrival) were purchased from Janvier SAS (France) and housed in a specific-pathogen-free barrier facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and handled in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. All experiments were approved by the local institutional animal care and use committee (BMBWF-66.006/0019-V/3b/2018). Mice were housed in groups under controlled conditions and had free access to tap water. All animals were adapted to the liquid control diet (C, 69E% carbohydrates, 12E% fat, 19E% protein, Ssniff, Germany) for 1 week prior to the experiment as explained in detail previously [22]. After adaption, mice randomly received the following diets and treatments for 7 weeks: mice fed a liquid C diet or a C diet enriched with antibiotics (AB, 1 g/L ampicillin, vancomycin, metronidazole, 160 mg/L gentamycin, [17]), a liquid fat- and fructose-rich diet (FFr, 60E% carbohydrates, 25E% fat, 15E% protein, Ssniff, Germany, detailed composition of diets see Supplemental Table 1) or a FFr + AB. The mixture of antibiotics was chosen based on previously published studies which assessed the contribution of intestinal microbiota in settings of steatohepatitis or hepatocellular carcinoma in mice and where it was reported that this antibiotic mixture almost completely depleted prevalence of bacteria [17,23]. No side effects such as diarrhea were detected. Body weight was assessed weekly and the C and FFr groups were pair-fed by adjusting the caloric intake daily to the group with the lowest caloric intake, respectively. In week 6 of the feeding trial, mice were fasted for 6 h and fasting blood glucose levels were assessed. At the end of the experiments, animals were anesthetized with a mixture of ketamine/xylazine (100 mg ketamine/kg BW; 16 mg xylazine/kg BW, i.p. injection). Blood was collected from portal vein just prior to killing and liver as well as intestinal tissue samples were collected

and snap-frozen or fixed in neutral-buffered formalin or, in case of intestinal tissue, were used for everted gut sacs as explained below. Sacrifice and all following measurements were performed in a mixed order of groups.

2.2. Everted gut sac and xylose permeation measurement

Naïve C57BL/6J male mice were killed by cervical dislocation and small intestine was rapidly removed and everted with a rod, described in more detail by others and illustrated in Fig. 3A [8,24]. Small intestinal tissue was cut in segments equally in length and put into cold DPBS. Each sac was knotted at both ends and filled with 1x Krebs-Henseleit-bicarbonate-buffer containing 0.2% (w/v) bovine serum albumin (KRH buffer). Experiment 1: Tissue sacs were challenged with +/- 5mM fructose (Fr) at 37°C in gassed KRH buffer (95% O₂/5% CO₂) incubated for 55 minutes (n=5). Experiment 2: After a preincubation of 10 minutes in gassed KRH buffer (95% O₂/5% CO₂) +/- antibiotic mixture (0.5 mg/mL ampicillin, vancomycin, metronidazole, 0.08 mg/mL gentamycin) at 37°C, tissue sacs were further incubated for 55 minutes at 37°C in gassed KRH buffer +/- 5mM fructose, in the presence or absence of the antibiotic mixture (n=5). Experiment 3: After preincubating tissue sacs for 10 minutes in gassed KRH buffer +/- 5mM inducible nitric oxide synthase (iNOS) inhibitor aminoguanidine hydrochloride [25] (Sigma-Aldrich, Germany) at 37°C, tissue sacs were treated as detailed above for the following 55 minutes in the presence of +/- fructose and +/- aminoguanidine (n=4). To assess tissue permeability after 55 minutes, 0.1% D-xylose (w/v; Merck KGaA, Germany) was added to the solutions in which tissue sacs were incubated for 5 minutes. As xylose permeation of control (C) sacs and C + aminoguanidine did not differ, only results of C are shown. In case of the feeding experiment (see section 2.1) everted sacs from the different treated mice were also incubated for 5 minutes in 0.1% (w/v) D-xylose. Intestinal tissue obtained from each sac was snap frozen for further analysis. For the detection of xylose in the samples, xylose concentration was assessed as detailed previously [8,26].

2.3. Histological evaluation of liver sections and inflammatory alterations

Liver was embedded in paraffin and tissue sections (4 µm) were stained with hematoxylin and eosin (Sigma-Aldrich, Germany). To evaluate the progression of the liver disease, NAFLD Activity Score (NAS) was used for scoring as described previously [27]. Neutrophil granulocytes were stained and counted in liver tissue sections using a commercially available kit (Naphthol AS-D Chloroacetate (Specific Esterase) Kit; Sigma-Aldrich, Germany) as explained before [12]. Thiobarbituric acid reactive substances (TBARS) in liver homogenate were assessed as previously described [4].

2.4. Blood parameters of liver damage and ligands for TLR4

TLR4 ligands in plasma of mice were assessed using a commercially available secreted embryonic alkaline phosphatase (SEAP) reporter HEK293 cell assay (Invivogen, France) as detailed previously [28]. Alanine aminotransferase (ALT) activity in plasma of mice was measured in a routine laboratory (Veterinary Medical University of Vienna, Vienna, Austria).

2.5. Detection of nitrite and arginase activity in small intestinal tissue

To assess nitrite levels in small intestinal tissue a commercially available Griess reagent kit was used according to the manufacturer's instructions (Promega, USA) and as detailed previously [8].

Arginase activity in small intestinal tissue was also assessed as previously described [8].

2.6. Immunohistochemical staining of zonula occludens-1 (ZO-1) and iNOS in small intestinal tissue

Paraffin-embedded sections (4 µm) of small intestinal tissue were used to determine ZO-1 and iNOS protein in mice as previously described [12,27,29]. In brief, sections were deparaffinized and treated with either protease (ZO-1) or heated in monosodium citrate buffer (iNOS), blocked with bovine serum albumin (iNOS) and incubated with the respective specific antibodies (iNOS: Thermo Fisher Scientific, USA, ZO-1: Invitrogen, USA). After using respective secondary antibodies linked with peroxidase, sections were incubated with diaminobenzidine (Peroxidase Envision Kit, DAKO, Germany) and the extent of iNOS staining was evaluated as previously explained [12].

2.7. Evaluation of goblet cell number

Small intestinal tissue sections embedded in paraffin (4 µm) were stained by Alcian Blue and periodic acid-Schiff staining, as described by others [30]. Using a microscope with an integrated camera (Leica DM4000 B LED, Leica, Germany), the number of goblet cells /100 µm villus was counted.

2.8. RNA isolation and real-time RT-PCR

RNA was extracted from liver tissue using Trizol (TRIzol, G, AppliChem, Germany) and after synthesization of cDNA (reverse transcription system obtained from Promega GmbH, USA), genes listed in Supplemental Table 2 were amplified with real-time polymerase chain reaction (PCR) as described in detail before and normalized to 18S [31].

2.9. Western blot

Protein was isolated from small intestinal tissue using Trizol as detailed previously [32]. After protein extraction, 30 and 10 µg protein were loaded on a polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, USA). Following 1 h blocking, membranes were incubated with the primary antibodies occludin (Invitrogen, USA) or β -actin (Santa Cruz, USA) overnight. After washing with 1X Tris-buffered saline with Tween and an incubation with the respective secondary antibody and a repeated washing, the bands were detected using Super Signal West Dura kit (Thermo Fisher Scientific, USA) as detailed previously [12]. Densitometric analysis of bands was performed using Software Image Lab 6.1 (BioRad Laboratories, USA).

2.10. Microbiota analysis

Samples from the small intestine were extracted as previously described [33] using the Trizol protocol (Trizol, Sigma Aldrich, Germany). The V3–V4 region of the 16S rRNA gene was targeted to prepare the sequencing libraries. Master mix was prepared using PrimeSTAR HS DNA Polymerase kit (TaKaRa, China) with 2 µl of DNA template, 0.2 µM Primer and 0.5 U Taq primer star HS DNA (TaKaRa, China) in a total volume of 25 µl for the first and second PCR and 50 µl for the third PCR. The PCR conditions comprised an initial denaturation at 95°C for 3 minutes, followed by 10 cycles for the first and second PCR and 20 cycles for the third PCR according to the protocol: 98°C denaturation for 10 s, annealing at 55°C for 10 s and extension at 72°C for 45 s followed by a final extension at 72°C for 2 minutes. Libraries were purified and standardized with

SequalPrep Normalization Kit (Invitrogen Inc., USA), and sequenced on an Illumina Novaseq platform. Total bacteria quantification was performed using the CFX Connect Real-Time PCR Detection System (BioRad Laboratories Inc., Germany). The qPCR reaction mixture consisted of 20 µL total volume, with 1 µL of each extraction combined with 10 µL of 2 × TB Green Premix Ex Taq (TaKaRa, China), and 0.2 uM of each primer (BAC338F (5'-ACTCCTACGGGAGGCAG-3') and BAC805R (5'-GACTACCAGGTATCTAATCC-3')).

Sequencing reads were processed using MOTHUR [34]. After quality filtering and chimeras removal by UCHIME, sequences were aligned against the database Silva version 138. Sequences from chloroplasts, mitochondria, archaea, and eukaryotes were deleted. Sample reads were standardized by total and a comparison between samples was made by creating a sample-similarity matrix using the Bray-Curtis similarity coefficient (Primer 7) [35]. For the visual hierarchical clustering and ordination of the community structures, a nonmetric multidimensional scaling (nMDS) plot was created using the package Vegan in R. Statistical differences between groups were tested using ANOSIM. The taxonomy graph was made using ggplot2 in R.

2.11. Statistical analysis

PRISM (Version 7.03, GraphPad Software, Inc.) was used for statistical analysis. Outliers were determined with Grubbs test. Data not homogenous in variance, was log-transformed. A two-factorial analysis of variance (ANOVA) was used to determine statistical differences between four groups followed by Tukey's *post hoc* test. In case of everted sac data paired Wilcoxon matched-pairs signed rank test or paired t-test was used. All data are presented as means $\pm$ standard error of the means (SEM). $P < .05$ was defined to be significant.

3. Results

3.1. Effect of antibiotics on the development of a diet-induced NAFLD, bacteria in feces, and fasting glucose in mice

As expected, and shown before by us [4,8,36], even in the absence of the development of overweight or obesity, the intake of a FFr diet resulted in the development of early signs of NASH in mice as determined by NAS and activity of ALT in plasma (Fig. 1A–B, Table 1). Furthermore, being also indicative of NASH development, mRNA expression of *Il6* and *F4/80* as well as number of neutrophils and concentration of TBARS were all significantly higher in liver of FFr-fed mice when compared to controls (Fig. 1C–E, Table 1). The development of NAFLD was associated with a decrease in evenness diversity of bacteria in small intestine of FFr-fed mice compared to C-fed mice (Fig. 2A), whereas Shannon's diversity did not differ between controls and FFr-fed mice (data not shown). The NMDS plot representing the microbial communities only differed by trend ($P = .1$) between groups. Still, the relative abundance of *Clostridiales* and *Oscillibacter* was significantly higher and lower, respectively, in small intestine of C-fed mice when compared to FFr-fed mice (see Fig. 2B–C and Supplemental Table 3).

In contrast, and while having gained more body weight despite being pair-fed, in FFr-fed mice concomitantly treated with AB, NAS and ALT activity in plasma as well as mRNA expression of *Il6* and *F4/80* mRNA, number of neutrophils and TBARS concentration in liver tissue were significantly lower than in FFr-fed mice. Indeed, levels were almost at the level of controls (Fig. 1A–E, Table 1). The protective effects of the antibiotic mix were associated with a reduction of >99% of bacteria in feces in FFr-fed mice treated with antibiotics (Table 2). Somewhat contrasting these findings, fasting glucose levels were similar between both FFr groups regardless

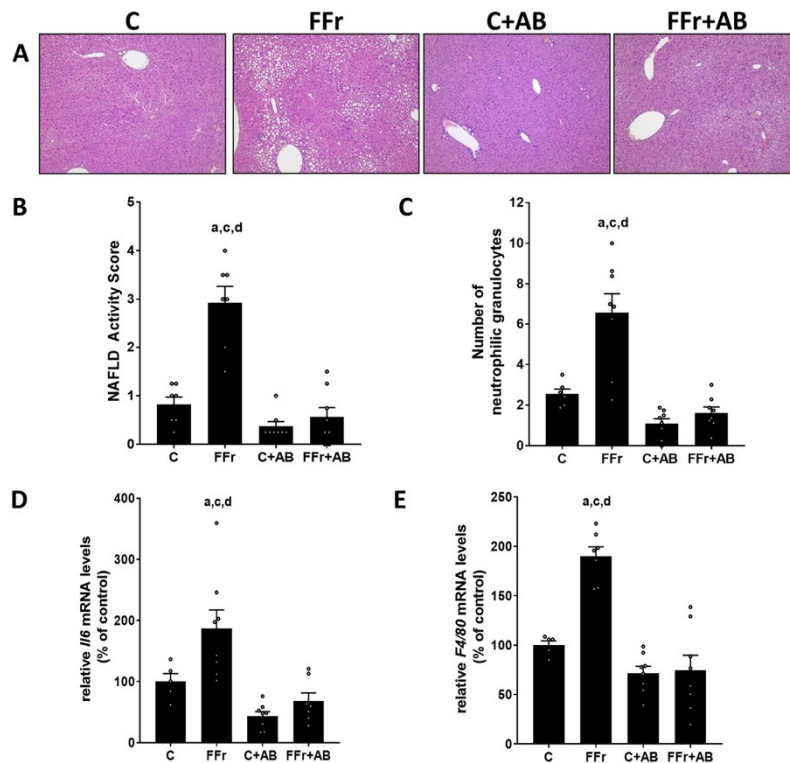


Fig. 1. Effect of antibiotics on the development of NAFLD in mice fed a fat- and fructose-rich diet. (A) Representative liver histology of sections stained with hematoxylin and eosin, (B) evaluation of liver damage using NAFLD Activity Score (NAS), (C) Number of neutrophil granulocytes per microscopic field in liver sections, (D) *Interleukin 6 (IL6)* and (E) *F4/80* mRNA expression in liver tissue of mice fed a liquid control (C) and fat- and fructose-rich diet (FFr) +/- antibiotic mixture (AB). Data are presented as mean $\pm$ sem. $n=5-8$. ^a $P<.05$ compared with mice fed a control diet, ^c $P<.05$ compared with mice fed a control diet enriched with antibiotics, ^d $P<.05$ compared with mice fed a fat- and fructose-rich diet enriched with antibiotics.

Table 1

Caloric intake, body weight and markers of liver damage and fasting glucose in mice fed a control or fat- and fructose-rich diet $\pm$ antibiotics enrichment

Parameter	Groups			
	C	FFr	C+AB	FFr+AB
Caloric intake [kcal/mouse/d]	9.0 $\pm$ 0.2	10.9 $\pm$ 0.1 ^{a,†}	8.6 $\pm$ 0.1	10.7 $\pm$ 0.2 ^{a,†}
Body weight [g]	25.9 $\pm$ 0.6	25.7 $\pm$ 0.5	25.9 $\pm$ 0.4	26.3 $\pm$ 0.4
Weight gain [g]	1.6 $\pm$ 0.4	1.9 $\pm$ 0.4	2.1 $\pm$ 0.5	3.1 $\pm$ 0.2 ^a
Liver: body weight ratio [%]	4.2 $\pm$ 0.1	5.0 $\pm$ 0.1 ^{a,†}	4.1 $\pm$ 0.0	4.9 $\pm$ 0.1 ^{a,†}
ALT [U/L]	19.3 $\pm$ 4.7	39.5 $\pm$ 6.4 ^{a,†,‡}	17.8 $\pm$ 0.8	14.3 $\pm$ 1.8
TBARS [μ M/ g protein]	94.5 $\pm$ 23	193 $\pm$ 28 ^{a,‡}	144 $\pm$ 17	107 $\pm$ 20
Fasting glucose [mg/dl]	104 $\pm$ 1.7	133 $\pm$ 4.0 ^{a,†}	104 $\pm$ 4.3	123 $\pm$ 2.6 ^{a,†}

Values are means $\pm$ sem.

Abbreviations: AB: Antibiotics; C: control diet; C+AB: control diet enriched with antibiotics; FFr: fat- and fructose-rich diet; FFr+AB: fat- and fructose-rich diet enriched with antibiotics; TBARS: Thiobarbituric acid reactive substances.

^a $P<.05$ compared with mice fed a control diet.

[†] $P<.05$ compared with mice fed a control diet enriched with antibiotics.

[‡] $P<.05$ compared with mice fed a fat- and fructose-rich diet enriched with antibiotics.

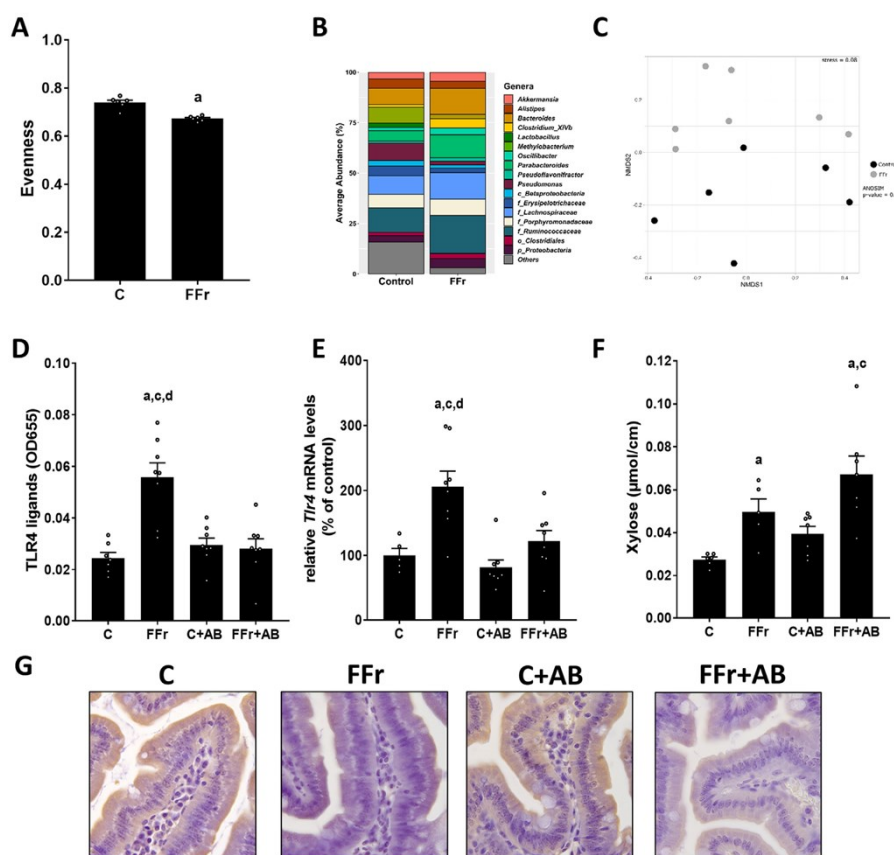


Fig. 2. Effect of antibiotics on small intestinal barrier in mice fed a control or fat- and fructose-rich diet. (A) Evenness, (B) stacked box plots representing the average abundance of the different genera and (C) nMDS representing the microbial communities in small intestine of C and FFr-fed mice. (D) Toll-like receptor 4 (TLR4) ligands in plasma of portal vein, (E) *Tlr4* mRNA expression in liver tissue, (F) xylose permeation assessed in everted sac of small intestinal tissue collected at the end of the feeding experiment and (G) representative staining of zonula occludens-1 (ZO-1) in small intestinal tissue (630 $\times$) of mice fed a liquid control (C) and fat- and fructose-rich diet (FFr) +/- antibiotic mixture (AB). c- class, f- family, o- order, p- phylum. Data are presented as mean $\pm$ sem, $n=5-8$. ^a $P<.05$ compared with mice fed a control diet, ^c $P<.05$ compared with mice fed a control diet enriched with antibiotics, ^d $P<.05$ compared with mice fed a fat- and fructose-rich diet enriched with antibiotics.

Table 2

Markers of intestinal barrier physiology and prevalence of bacteria in feces of mice fed a control or fat- and fructose-rich diet $\pm$ antibiotics enrichment

Parameter	Groups			
	C	FFr	C+AB	FFr+AB
goblet cells/ 100 μ m villus	4.8 $\pm$ 0.2	5.0 $\pm$ 0.3	5.3 $\pm$ 0.3	4.9 $\pm$ 0.4
relative <i>Mucin 2</i> mRNA levels	100 $\pm$ 25	102 $\pm$ 12	98.1 $\pm$ 8.3	150 $\pm$ 24
copy number q PCR feces	9.1E+08 $\pm$ 2.8E+08	1E+08 $\pm$ 4E+07	n.d.	n.d.

Values are means $\pm$ sem, mRNA expression expressed as % of C.

Abbreviations: AB: Antibiotics; C: control diet; C+AB: control diet enriched with antibiotics; FFr: fat- and fructose-rich diet; FFr+AB: fat- and fructose-rich diet enriched with antibiotics; n.d.: not detectable.

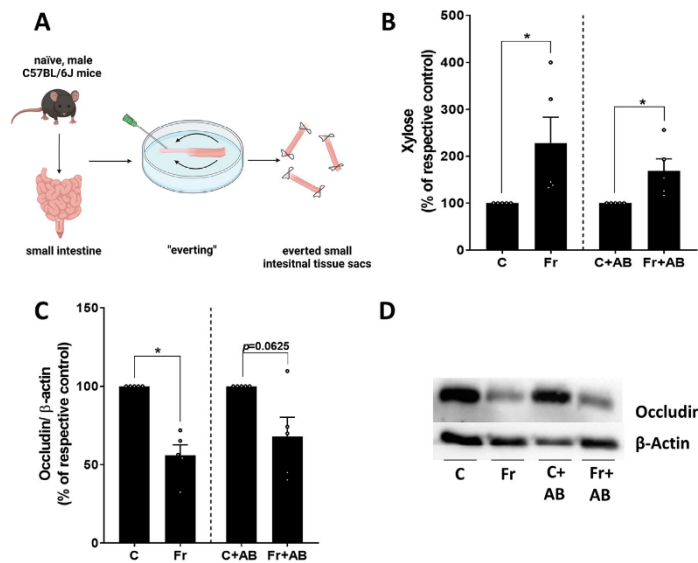


Fig. 3. Effect of antibiotics and fructose on intestinal barrier function in small intestinal everted tissue sacs. (A) Schematic drawing of creating a small intestinal everted tissue sac. (B) xylose permeation and (C) protein levels of occludin and (D) representative blots in everted gut sacs of intestinal tissue incubated with KRH buffer in the presence of 0 and 5mM fructose (C and Fr) and +/- antibiotics (AB). Data are presented as mean $\pm$ sem, $n=5$. * $P<.05$. Fig. 3A created with BioRender.com.

of additional treatments being significantly higher in both groups than in C-fed groups (Table 1).

3.2. Effect of antibiotics on TLR4 ligands in portal blood and Tlr4 mRNA expression in liver tissue as well as on markers of intestinal barrier function

In line with previous findings of us and others [4,8,10,13], concentration of TLR4 ligands e.g., bacterial lipopolysaccharide in portal blood and expression of *Tlr4* mRNA in liver were significantly higher in FFr-fed mice than in all other groups. As expected, concentration of TLR4 ligands in portal blood and mRNA expression of *Tlr4* in liver tissue were at the level of controls in FFr+AB-fed mice further suggesting that the treatment with AB successfully diminished intestinal microbiota (Fig. 2D–E). Somewhat contrasting these findings, xylose permeation was significantly higher in both FFr-fed groups irrespective of the addition of AB when compared to C-fed group. Interestingly, intestinal permeability was also significantly higher in C+AB-fed mice when both groups were compared (students t-test) (Fig. 2F). In line with these findings, protein staining of ZO-1 was also lower in both FFr-diet groups when compared to respective controls (representative staining see Fig. 2G). However, neither gross morphology (data not shown) nor number of goblet cells/ 100 μ m villus or *mucin 2* mRNA expression in small intestine differed between groups (Table 2).

3.3. Effect of antibiotics and fructose on intestinal permeability in small intestinal tissue in an ex vivo model

To further delineate the role of bacteria and/ or diet on the development of intestinal barrier dysfunction, we challenged everted intestinal tissue sacs with fructose in physiological relevant

concentrations in the presence or absence of AB. In line with previous findings [8,37], xylose permeation in tissue sacs challenged with fructose was significantly higher than in tissue sacs only incubated in KRH buffer. The addition of AB to the incubation buffer had no effects on the fructose-induced increased xylose permeation (Fig. 3B). In line with these findings, protein levels of occludin were also found to be decreased to a similar extent in both fructose and fructose +AB incubated everted tissue sacs compared to control tissue sacs (Fig. 3C–D).

Recently, we have shown that fructose-induced alterations of intestinal barrier function in small intestinal tissue may be related to changes in nitric oxide (NO)-homeostasis [8,37]. Indeed, NO_x levels in fructose-challenged everted tissue sacs were almost double compared to controls, while activity of arginase, being described as a counterplayer of iNOS [38], was significantly lower than in control sacs (Fig. 4A–B). Furthermore, supporting a critical role of NO-homeostasis in the development of fructose-induced barrier dysfunction, in tissue sacs pretreated with the NOS inhibitor aminoguanidine before exposed to fructose +/- aminoguanidine, permeability of xylose was reduced to the level of controls. This was also associated with lower levels of nitrite in these sacs (Fig. 4C–D). In line with these findings, in FFr-fed mice *in vivo*, iNOS protein concentrations assessed by immunohistochemical staining in small intestine was also higher in both FFr-fed groups, regardless of additional treatment compared to C-fed groups (Fig. 4E and Supplemental Fig. 1). Furthermore, in small intestinal tissue of FFr-fed mice, nitrite levels were significantly higher than in both control groups but also than in FFr+AB fed mice. In small intestinal tissue of FFr+AB-fed mice nitrite levels were also by trend higher than in the respective C-fed mice ($P=.052$). However, nitrite levels were per se lower in AB-fed animals than in mice not receiving AB (C-fed vs. C+AB-fed mice $P<.05$, students t-test) (Fig. 4F).

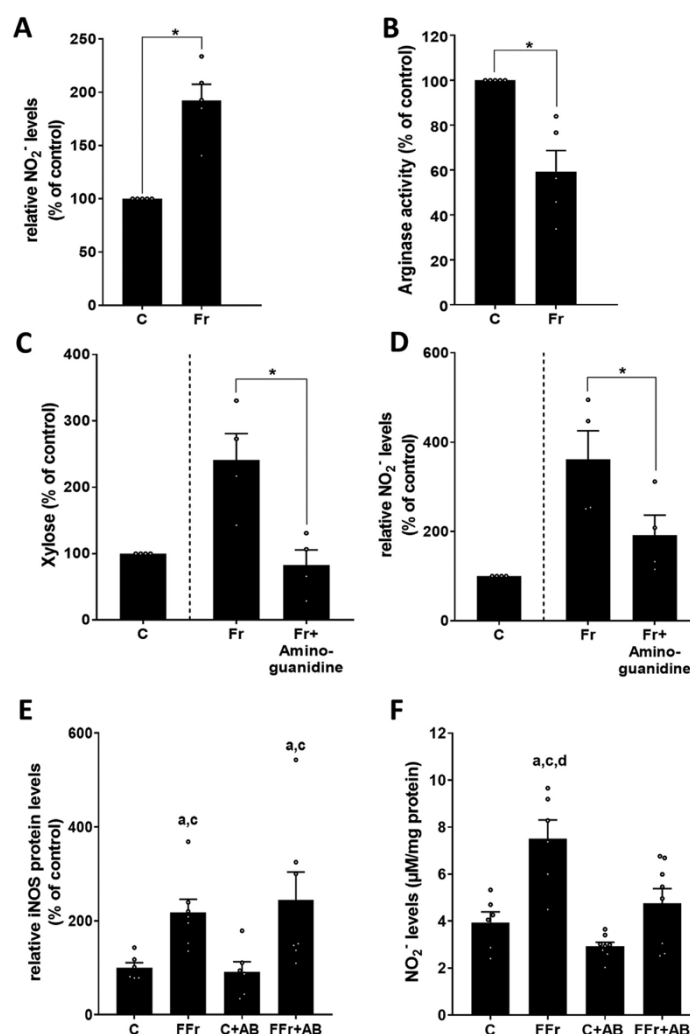


Fig. 4. Role of NO homeostasis in fructose- and diet-induced intestinal barrier dysfunction. (A) NO₂⁻ concentration and (B) arginase activity in small intestinal everted tissue sacs incubated with KRH buffer in the presence of 0 and 5mM fructose (C and Fr) ($n=5$). (C) Xylose permeation and (D) NO₂⁻ concentration in small intestinal everted tissue sacs incubated with KRH buffer in the presence of 0 and 5mM fructose (C and Fr) and aminoguanidine ($n=4$). (E) Densitometric analysis of inducible nitric oxide synthase (iNOS) protein staining in small intestine and (F) NO₂⁻ concentration in small intestine of mice fed a liquid control (C) and fat- and fructose-rich diet (FFr) +/- antibiotic mixture (AB) ($n=6-8$). Data are presented as mean $\pm$ sem. * $P<.05$, ^a $P<.05$ compared with mice fed a control diet, ^c $P<.05$ compared with mice fed a control diet enriched with antibiotics, ^d $P<.05$ compared with mice fed a fat- and fructose-rich diet enriched with antibiotics.

4. Discussion

By now, an interaction of alterations of intestinal microbiota composition, an increased translocation of bacterial endotoxin and the development of NAFLD has been established (for overview see [39]). Indeed, results of animal studies suggest that through a transfer of fecal microbiota from humans or mice the NAFLD phenotype can be transferred between species [40,41]. However, molecular mechanisms underlying the loss of intestinal barrier

function e.g., if the change of intestinal microbiota composition directly or through indirect measures affects intestinal barrier function, has not yet been clarified. In line with studies of others in humans and rodent models of NAFLD [4,6,7,42], in the present study, diversity of intestinal microbiota composition was markedly decreased and shifted in relative abundance of several bacterial species. Employing a pair-feeding mouse model of diet-induced NAFLD, here we have shown that a concomitant treatment of mice fed a fructose- and fat-rich diet with an antibiotic mix protected

mice from the development of early signs of NAFLD e.g., the accumulation of fat in the liver, infiltration of proinflammatory cells and increased expression of proinflammatory cytokines. This is in line with previous findings of our group and of others reporting that in rodents the depletion of microbiota with antibiotic is associated with a protection from the development of fructose- and/or fat-rich diet-induced NAFLD [15,16,43,44]. Also, results obtained in germ-free mice fed a fat- and fructose-rich diet suggest that a loss of intestinal microbiota protects mice from the development of NAFLD [45]. Contrasting our findings and those of others [15,16,43,44], Schneider et al. reported an exacerbation of the development of a methionine choline deficient diet (MCD) induced NAFLD in mice treated with an almost identical antibiotics mix (0.5 g/L neomycin, 1 g/L metronidazol, 1 g/L vancomycin, 1 g/L ampicillin) [19]. Differences between the study of Schneider et al. and our study could be related to marked differences in the feeding model (here high fructose high fat diet vs. MCD lacking the metabolic features typically associated with NAFLD). Somewhat contrasting the protective effects found for the development of NAFLD, the concomitant treatment of mice fed the FFr diet with the antibiotics had no effects on fasting glucose levels being markedly higher in both FFr-fed mouse groups compared C-fed groups. Results regarding the effects of antibiotics on the development of insulin resistance in settings of NAFLD are contradictory (for overview see [46]). Indeed, while some studies report that the concomitant treatment of antibiotics attenuated both, the development of NAFLD and insulin resistance [44] others found no effect [47] or no persistent effects [48]. Also, effects of different antibiotics regarding blood glucose levels have been reported to be strain specific [49]. As in the present study, a mix of antibiotics was used, it cannot be ruled out that the lack of protection against the increase of fasting glucose level might also, at least in part, have resulted from an interaction of diet and the antibiotics or associated adverse effects. Taken together, results of the present study further bolster the hypothesis (1) that the development of NAFLD is associated with alterations of intestinal microbiota composition and (2) that a diminishment of intestinal microbiota >90% can diminish the development of NAFLD irrespective of the diet fed while having no or only limited effects on the development of insulin resistance. However, our data by no means preclude that when taken in lower doses or diminishing only selected bacterial species, the development of diet-induced NAFLD may even be enhanced in settings of antibiotic treatment.

4.1. Impairments of intestinal permeability in small intestine are not affected by the lack of bacteria but rather seems to be associated with direct effects of fructose

Results of several studies suggest that not only a change in intestinal microbiota composition but also impairments of intestinal barrier function may be critical in the development of NAFLD in both humans and rodents [4,6–8]. Indeed, we and others have shown that the development of NAFLD is associated with a loss of tight junction proteins in small intestine [6,7,12,13]. Furthermore, it also has been shown that interventions targeting the loss of tight junction proteins and subsequently increased translocation of bacterial endotoxin may prevent the development of NAFLD even in the absence of marked changes in intestinal microbiota composition [8]. In the present study, the depletion of intestinal microbiota with antibiotics in FFr-fed mice was neither associated with a protection against the loss of tight junction proteins nor the increased permeation of xylose found in FFr-fed mice. Rather, permeation of xylose and concentration of the tight junction protein occludin were similarly altered in small intestinal tissue of FFr+AB fed mice as in FFr-fed animals while concentration of TLR4 ligands

e.g., bacterial endotoxin, were almost at the level of controls. In line with these findings, the concomitant treatment with AB of everted small intestinal tissue sacs challenged with fructose was not associated with a protection against the loss of tight junction protein and increase in xylose permeation.

Recently, we showed that a chronic intake of a fructose-rich diet and the acute *ex vivo* exposure of small intestinal tissue to fructose is associated with a loss of tight junction proteins and increased permeability of the tissue in mice [8,37]. In these studies, the loss of intestinal barrier function was associated with a decrease in arginase activity and enhanced formation of NO_x. Enhancing arginase activity with its allosteric regulator citrulline and arginine, respectively, attenuated the effects of fructose on intestinal permeability [8,37]. Indeed, arginase has been shown to be a counter regulator of iNOS thereby being critical in maintaining NO-homeostasis suggested before to be important in maintaining intestinal barrier function [8,37]. In line with these findings, in the present study, we found that in mice fed the FFr regardless of additional treatment with antibiotics, iNOS protein levels were induced in small intestinal tissue being associated with higher levels of NO_x. Furthermore, the fructose-induced impairments of intestinal barrier function in small intestinal everted tissue sacs were associated with a decrease in arginase activity and increase in NO_x levels, and were attenuated in the presence of the iNOS inhibitor aminoguanidine. Taken together, these data suggest that even in the absence of intestinal microbiota the chronic intake of a fat- and fructose-rich diet is associated with impaired small intestinal barrier function. Our data further suggest that fructose, either through a direct interaction with iNOS and arginase or through its metabolites can affect NO-homeostasis and subsequently intestinal barrier function. Based on our findings, besides recommending to reduce the intake of free sugar (e.g. sucrose, high-fructose corn syrup), manipulating intestinal NO homeostasis through specific iNOS inhibitors or arginase activators may be a therapeutic strategy to prevent diet-induced NAFLD. Indeed, results of our own studies in mice suggest that supplementation of a fructose-rich diet with L-citrulline or L-arginine in pharmacological doses may attenuate the loss of arginase activity in small intestine and the development of diet-induced NAFLD [8,37], while it still needs to be clarified if the iNOS inhibitor aminoguanidine exerts similar effects in mice.

5. Conclusion

In summary, results of our study further bolster the hypothesis that intestinal microbiota and the translocation of bacterial compounds like bacterial endotoxin are critical in the development of NAFLD. However, results of our study also suggest that while changes in intestinal microbiota composition and an increased translocation of bacterial wall compounds like endotoxin, associated with the development of diet-induced NAFLD, are critical in the development of liver injury, these alterations may not be the key triggers of intestinal barrier dysfunction. Rather, results of the present study further add weight to the hypothesis that a high intake of certain nutrients like fructose may directly alter intestinal barrier function through interaction either of the nutrient or metabolites derived from it with the enterocytes. Further studies are warranted to determine molecular mechanisms underlying this interaction of fructose with small intestinal enterocytes as well as to determine, if alterations alike are also found in humans consuming large amounts of fructose.

Institutional review board statement

The animal study protocol was approved by local institutional animal care and use committee (BMBWF-66.006/0019-V/3b/2018).

Data availability

Data will be made available on reasonable request. Sequencing data were submitted to the European Nucleotide Archive under the accession number PRJEB62145.

Declaration of competing interest

All authors declare no conflict of interest.

CRediT authorship contribution statement

Annette Brandt: Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Katja Csarman:** Formal analysis, Investigation, Visualization, Writing – review & editing. **Angélica Hernández-Arriaga:** Formal analysis, Investigation, Visualization, Writing – review & editing. **Anja Baumann:** Investigation, Writing – review & editing. **Raphaela Staltner:** Investigation, Writing – review & editing. **Emina Halilbasic:** Conceptualization, Writing – review & editing. **Michael Trauner:** Conceptualization, Funding acquisition, Writing – review & editing. **Amélia Camarinha-Silva:** Formal analysis, Visualization, Funding acquisition, Writing – review & editing. **Ina Bergheim:** Conceptualization, Supervision, Funding acquisition, Writing – original draft, Writing – review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2023.109495.

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2.2. Publication 2: Fructose intake enhances lipoteichoic acid-mediated immune response in monocytes of healthy humans

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Highlights:

- Intake of fructose, in contrast to glucose, increased TLR2 mRNA expression in human monocytes.
- After fructose intake, isolated immune cells showed a significantly increased LTA-induced release of pro-inflammatory cytokines
- Blocking fructose metabolism prevented both the upregulation of TLR2 and the increased cytokine release, an indication of a direct metabolic effect of fructose.
- Inhibition of SP1 reduced fructose-dependent TLR2 expression and cytokine response, identifying SP1 as a central mediator of fructose-enhanced immune responses.

Contribution to the manuscript:

- Laboratory chemical analyses of human blood samples
- Writing – review and editing

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Fructose intake enhances lipoteichoic acid-mediated immune response in monocytes of healthy humans

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ABSTRACT

Metabolic diseases like type 2 diabetes are afflicted with higher rates of infections and longer, more complicated infection course as well as higher fatality rates. The impact of nutrition and specific nutrients like free fructose herein has not yet been fully understood. Here, we performed dietary intervention studies in healthy individuals and performed *ex vivo* experiments in isolated blood immune cells to assess the effects of dietary fructose intake on Gram-positive bacterial toxin induced immune responses. Acute and extended intake of fructose but not glucose was related with an induction of *Toll like receptor 2* mRNA expression in monocytes and enhanced the LTA-dependent release of proinflammatory cytokines from monocytes. Blocking fructose metabolism and transcription factor SP1 attenuated the fructose-related induction of *Toll like receptor 2* mRNA expression and augmentation of proinflammatory cytokine release further suggesting that fructose-dependent metabolic alterations are critical in enhancing immune responsiveness of humans after fructose consumption.

1. Introduction

Despite significant advance in vaccination and the development of therapeutics like antibiotics, infectious diseases caused by bacteria and viruses are still among the leading causes of deaths world-wide [1]. Indeed, it has been estimated that in 2019, 7.7 million deaths around the world were accounted to bacterial infections [2]. Taken all global deaths in consideration, this implies that 13.6 % or 1 in 8 deaths is related to bacterial infections. Studies further identified 33 bacterial pathogens across 11 infectious syndromes to be associated with bacterial infection related deaths [2]. Among these bacterial pathogens five bacteria including *Staphylococcus aureus*, *Streptococcus pneumoniae* accounted to the Gram-positive bacteria and *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* accounted to the Gram-negative bacteria are responsible for more than 50 % of deaths among the investigated bacteria. In the sub-Sahara Africa super-region, higher population, poor housing, and poor sanitary infrastructure and a lack of medical care are among the key risk factors for bacterial infections and their fatality outcome [3]. In contrast, in industrialized countries it has been shown

that metabolic diseases like metabolic dysfunction-associated steatotic liver disease and type 2 diabetes are afflicted with higher rates of infections, a longer and more complicated infection course and a higher fatality rate compared to healthy individuals [4,5]. Indeed, it has been discussed that the low-grade inflammation associated with metabolic diseases may gradually damage for instance liver tissue and subsequently, its immune function⁴. However, underlying mechanisms have not yet been clarified. It has also been suggested that a diet rich in certain nutrients like saturated fats and fructose may be critical in the development of metabolic diseases [6,7]. For instance, while still being discussed controversially with respect to the nutritional source of the sugar e.g., total fructose intake vs. fructose intake through sugar sweetened beverages [8], excessive fructose intake has been linked to an increased risk of metabolic dysfunction-associated steatotic liver disease (MASLD), cirrhosis, and hepatocellular carcinoma [9]. This has been accounted primarily to an increased hepatic *de novo* lipogenesis and reduced fatty acid oxidation, which promote liver fat accumulation and insulin resistance [10]. Also, fructose metabolism may lead to elevated uric acid levels and oxidative stress, further aggravating metabolic

Abbreviations: IL, interleukin; KHK, ketoheoxokinase; LTA, lipoteichoic acid; PAMP, pathogen associated molecular pattern; PBMC, peripheral blood mononuclear cells; TLR, Toll-like receptor; TNF α , tumor necrosis factor alpha.

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disturbances (for overview see Ref. [11]). Moreover, studies suggest that this “Western diet” may not only increase the odds to develop non-communicable but also infectious diseases (for overview see Refs. [12–14]). Studies have also suggested that free fructose may enhance immune response to bacterial endotoxin [15]. In line with these findings, our own studies in healthy young adults suggest that an intake of high doses of free fructose is associated with increased blood levels of bacterial endotoxin and an induction of *Toll like receptor (TLR) 2* and 4 mRNA expression in peripheral blood mononuclear cells (PBMCs). Interestingly, a similar alteration of *TLR2* and 4 mRNA expression was not found after the intake of similar doses of free glucose [16]. However, whether this induction of TLRs found after the intake of elevated amounts of free fructose is primarily a result of the monosaccharide intake or the associated elevation of bacterial (endo)toxin levels in peripheral blood and if this induction of TLRs also affect immune response has not yet been clarified.

Starting from this background, the aim of the present study was to determine the effects of fructose consumption on TLR2 signaling in blood immune cells in healthy humans upon a challenge with its ligand Lipoteichoic acid (LTA) and to assess underlying molecular mechanisms.

2. Material and methods

2.1. Intervention studies

Intervention studies were carried out in accordance with the Declaration of Helsinki (revised Version 1983) and were approved by ethics committees of the University of Vienna, Vienna, Austria (reference number: 00184) and the University Hospital Jena, Jena, Germany (reference number: 4588-11/15). All participants gave informed consent before being enrolled in the study. The human intervention studies were registered at clinical trials (NCT04788680, NCT03482284). For both studies the following exclusion criteria were defined: (1) following a special diet or (2) food malabsorption or (3) a history of diseases of the gastrointestinal tract. Furthermore, participants should be of normal-weight (BMI >18.5 kg/m² or <24.9 kg/m²) and not drink more than moderate amounts of alcohol (<10 g/day for women; <20 g/day for men) nor smoke.

All interventions were carried out in a randomized cross-over design. Before the challenges with the different sugars dietary intake of participants enrolled in the two studies was standardized to a ‘healthy nutrition’ being in accordance with the recommendations of the German, Austrian and Swiss nutrition societies (DACH) for 2 and 4 days, respectively, as described in detail before [16,17]. In brief, after assessing dietary intake by two independent 24-h recalls, participants were asked to follow a fully balanced diet with all foods and beverages provided for 2 and 4 days, respectively, that was isocaloric to their ‘normal’ diet. In **study A**, participants were asked to provide a fasting blood sample to determine clinical parameters in serum. After the 4-day long standardization of their nutritional intake another fasting blood sample was collected and participants received either a fructose or glucose rich diet (25 % of the total daily energy intake) where both sugars were provided as jellies for another 3 days, respectively. After each intervention a further fasting blood draw was collected. For further details please also see Nier et al. [16]. PBMCs were isolated from whole blood and fructose levels in plasma were assessed as detailed below.

In **study B**, a fasting blood sample was collected before subjects were standardized in their nutritional intake to assess clinical parameters in serum. After being standardized in their nutritional intake for 2 days and an overnight fast, fasting blood was drawn. Participants were then asked to consume the respective sugar containing beverage within 1 h in combination with a light breakfast (a roll and 10 g butter). Additional blood samples were collected 120 min after the consumption of the respective beverages, which were used to isolate monocytes and to carry out stimulation experiments and to determine plasma levels of fructose as described in detail below. The study beverages consisted either of

fructose (110 g) or maltodextrin (110 g) dissolved in 1 L of sparkling tap water. Doses were based on the assumption that similar amounts of sugar are found in 1 L sugar-sweetened beverages. Maltodextrin was selected over glucose to increase compliances as in pilot experiments it was found that the intake of less sweet beverages is favored by especially healthy subjects. Intake and standardization of the nutrition of the healthy subjects of both studies was assessed and calculated using the computer software EBISpro (Version 2011, Willstätt, Germany). For basic characteristics of the study subjects of **study A** and **B** see [Table S1](#) and [S2](#). For results of 24-h recalls and standardization of nutrition of **study B** see [Table S3](#).

2.2. Isolation of PBMCs, monocytes and B- as well as T-cells

PBMCs, monocytes and B- as well as T-cells, respectively, were either isolated from whole blood samples obtained from participants of **study A** and **B** or from healthy donors, and buffy coats. Buffy coats for the isolation of cells were obtained from the ‘Austrian Rotes Kreuz’. In addition, after obtaining written informed consent and an overnight fast, blood was drawn from healthy, normal weight donors which was approved by the ethics committees of the University of Vienna, Vienna, Austria (reference number: 00586). Cells were isolated by density gradient centrifugation using *pluriSelect* system following the instructions of the manufacturer (*pluriSelect* Life Science UG & Co. KG, Leipzig, Germany). In brief, blood or buffy coats were layered onto monocytes density gradient media (1.069 g/ml) in *pluriSelect* separation tubes and centrifuged at room temperature for 15 min for isolating monocytes. PBMCs were isolated by layering blood onto *Pancoll* solution (1.077 g/ml) and centrifugation for 30 min at room temperature. B- and T-cells were obtained in a second step by layering PBMC fraction onto an iso-osmotic *Percoll* solution (46 %) and centrifugation for 30 min at room temperature. Cells were collected and used for further experiments (see below).

2.3. Stimulation of monocytes obtained after an oral challenge with fructose or maltodextrin with a TLR2 ligand

After consumption of the test beverage in **study B**, isolated monocytes were seeded in 24-well plates in RPMI medium (RPMI-1640-Medium without glucose, with L-glutamine and sodium bicarbonate; Sigma Aldrich, Darmstadt, Germany) containing 10 % fetal bovine serum (Pan-Biotech GmbH, Aidenbach, Germany) and 100 µg/ml streptomycin and 100 U/ml penicillin (Pan-Biotech GmbH, Aidenbach, Germany) in a 5 % carbon dioxide atmosphere. Monocytes were then challenged with or without lipoteichoic acid (LTA, 10 µg/ml, derived from *Staphylococcus aureus*, Sigma-Aldrich) for additional 2 h. LTA is a component of the wall of Gram-positive bacteria including *Staphylococcus aureus* and acts as a pathogen-associated molecular pattern (PAMP) binding to TLR2 and leading to an activation of dependent signaling cascades [18]. Cells and supernatant of cells were collected for measuring mRNA expression and protein concentration of cytokines (see below).

2.4. Experiments in PBMCs, monocytes as well as B- and T-cells isolated from buffy coats and healthy donors

Isolated PBMCs, monocytes as well as B- and T-cells were seeded in 24 well plates by using RPMI medium (RPMI-1640-Medium without glucose, with L-glutamine and sodium bicarbonate; Sigma Aldrich, Darmstadt, Germany) containing 10 % fetal bovine serum (Pan-Biotech GmbH, Aidenbach, Germany) and 100 µg/ml streptomycin and 100 U/ml penicillin (Pan-Biotech GmbH, Aidenbach, Germany) in a 5 % carbon dioxide atmosphere. In a **first set of experiments**, PBMCs were treated with fructose (50 µM) for 2, 6 and 24 h while monocytes as well as B- and T-cells were treated with fructose (50 µM) for 2, 6 or 12 h. For assessing the mRNA expression of proinflammatory cytokines, monocytes were treated with fructose (50 µM) for 12 h followed by stimulation with LTA

(10 $\mu\text{g/ml}$) for additional 2 h in a **second set of experiments**. Furthermore, in a **third set of experiments**, monocytes treated with fructose (50 μM) were concomitantly treated with the SP1 inhibitor MIT (mithramycin, 10 nM) or the KHK inhibitor (KHK inhibitor 1, N8-(Cyclopropylmethyl)-N4-(2(methylthio)phenyl)-2-(1-piperazinyl)-pyrimidinol[5,4-d]pyrimidine-4,8-diamine (1 μM)) for 12 h. In a **fourth set of experiments** monocytes were treated with fructose (50 μM) and the KHK inhibitor (1 μM) for 12 h and further incubated with LTA (10 $\mu\text{g/ml}$) for 2 h. Supernatant and cells were collected after the incubation, respectively for further analysis.

2.5. *Caco-2 trans-well model*

The study design is summarized in Fig. 3a. Differentiated Caco-2 cells (ACC 169, DSMZ, Braunschweig, Germany) were grown in *trans*-wells by using DMEM medium containing 10 % fetal bovine serum (Pan-Biotech GmbH, Aidenbach, Germany) and 100 $\mu\text{g/ml}$ streptomycin and 100 U/ml penicillin (Pan-Biotech GmbH, Aidenbach, Germany) in a 5 % carbon dioxide atmosphere. Monocytes were seeded in the basolateral compartment of the *trans*-well system. In a **first set of experiments**, after reaching confluency, Caco-2 cells were treated apical with fructose (10 mM) for 14 h and collected for further assessment of GLUT5 protein concentration. Furthermore, in a **second set of experiments**, after Caco-2 wells were exposed to fructose (10 mM) for 14 h, human monocytes in the basolateral compartment were then stimulated with LTA (10 $\mu\text{g/ml}$) for 2 h. Caco-2 cells and monocytes were lysed in TRI-tyd G (peqGOLD Trifast, Peqlab, Germany) for subsequent analysis.

2.6. *Fructose assay*

Fructose concentration was measured in plasma of human participants of **study A** and **study B** as well as in the basolateral compartment of the Caco-2 *trans*-well using a commercially available fructose assay kit (Abnova GmbH, Heidelberg, Germany).

2.7. *ATP assay*

Monocytes isolated from buffy coats were lysed in a buffer consisting of 50 mM Tris HCl, 150 mM NaCl, 2 mM EDTA, 1 % Nonidet P40. ATP levels were measured using a commercially available cellTiter-Glo Luminescent Cell Viability Assay (Promega GmbH, Walldorf, Germany) following the instructions of the manufacturer.

2.8. *RNA extraction and real-time PCR*

RNA was extracted from monocytes, B- and T-cells and PBMCs using TRI-tyd G (peqGOLD Trifast, Peqlab, Germany) and reverse transcribed using a cDNA synthesis kit (Promega, Walldorf, Germany) following the instructions of the manufacturer. Primers used to determine *GLUT5*, cytokines (*TNF α* , *IL-1 β* , *IL-6*), Toll like receptors (*TLR1*, 2, 6), ketohe-oxinase (*KHK*) and *SP1* mRNA expression were designed using the software Primer 3. To determine the amount of target genes, normalized on endogenous reference (*18S*) and relative to a calibrator ($2^{-\Delta\Delta\text{Ct}}$), the comparative CT method was used as detailed previously. Primer sequences are shown in Table S4.

2.9. *Enzyme-linked Immunosorbent assay (ELISA)*

Protein concentrations of IL-1 β , IL-6, TNF- α , and soluble TLR2 were analyzed in cell culture supernatant of the monocyte *in vitro* experiment as well as of the human intervention **study B** using commercially available ELISA kits (Bio-Techne Corp., Minneapolis, MN, USA) according to the manufacturer.

2.10. *Western blot analysis*

To obtain total protein, Caco-2 cells and monocytes were lysed in TRI-tyd G (ITW Reagents, S.R.L, Milano, Italy) according to the instructions of the manufacturer. Protein was then separated in a gel electrophoresis and transferred onto a PVDF membrane. Membranes were then incubated with anti-GLUT5, anti-SP1 (Santa Cruz Biotechnology, Dallas, TX, USA), or anti- β -actin (Santa Cruz Biotechnology, Dallas, TX, USA) at 4 °C overnight, and subsequently incubated with a secondary antibody (anti-mouse or anti-rabbit, respectively Cell Signaling Technology, Danvers, MA, USA). Band intensity was analyzed with Super Signal West Dura Kit (Thermo Fisher Scientific, Waltham, MA, USA) and densities of the bands were analyzed with ChemiDoc XRS system as previously described [19].

2.11. *Statistical analysis*

Data are presented as mean $\pm$ SEM. Grubb's test was performed before statistical analysis to identify outliers. Normality of distribution of data was analyzed using Shapiro-Wilk normality test. Differences between two paired groups were assessed using a paired T-Test for each time point, respectively. Data were log-transformed if not normally distributed. All data were analyzed with GraphPad Prism software (GraphPad Prism Software Inc., USA). A p-value ≤ 0.05 was considered significant.

3. Results

3.1. *Effect of fructose on TLR2 and GLUT5 expression in PBMCs, monocytes, B- and T-cells*

To determine if the induction of *TLR2* mRNA expression in PBMCs found previously in healthy subjects consuming a fructose enriched diet [16] was a result of the sugar itself and in which subfraction of PBMCs *TLR2* mRNA expression was induced, we stimulated monocytes, B- and T cells isolated from buffy coats with fructose (50 μM) for 2, 6, and 12 h (for experimental set-up see Fig. 1a). Expression of *TLR2* mRNA was significantly induced in monocytes exposed to fructose after 6 and 12 h while no changes in *TLR2* mRNA were found after 2 h when compared to naïve cells (Fig. 1b). Similar effects were not found when cells were exposed to a glucose enriched cell culture media (see Fig. S1). Also, expressions of *TLR1* and 6 mRNA were not altered in monocytes exposed to fructose at any of these timepoints (see Table 1). Furthermore, *TLR2* mRNA expression was below the level of detection in B- and T-cells regardless of additional treatments (Fig. 1b).

To determine if the fructose-dependent induction of *TLR2* was related to changes in fructose uptake and metabolism in PBMCs and monocytes, respectively, samples from healthy subjects that were asked to consume fructose (25 E%) or glucose (25 E%) in a cross-over design study were analyzed (study design Fig. 1c and Table S1 as well as Nier et al. [16]). Three hours after the intake of fructose a significant post-prandial increase in serum fructose levels was observed (Fig. 1d). Moreover, compared to baseline, glucose transporter 5 (*GLUT5*) mRNA expression in PBMCs was induced by ~ 3 -fold after subjects had consumed the fructose enriched diet for 3 days (Fig. 1e). A similar increase in *GLUT5* mRNA expression in PBMCs was not found after the intake of the glucose enriched diet in which all other foods and beverages were similar to those consumed when subjects were exposed to fructose (Fig. 1d and e).

Furthermore, challenging PBMCs *ex vivo* with fructose for 2, 6, and 24 h resulted in a significant and by trend higher induction of *GLUT5* mRNA expression after 6 h ($p \leq 0.05$) and 24 h ($p = 0.08$) compared to naïve controls (Fig. 1f). To further determine in which cell population *GLUT5* mRNA expression was altered, we next challenged monocytes and B- as well as T-cells with fructose for 2, 6, and 12 h. *GLUT5* mRNA expression was only detectable in monocytes and was significantly

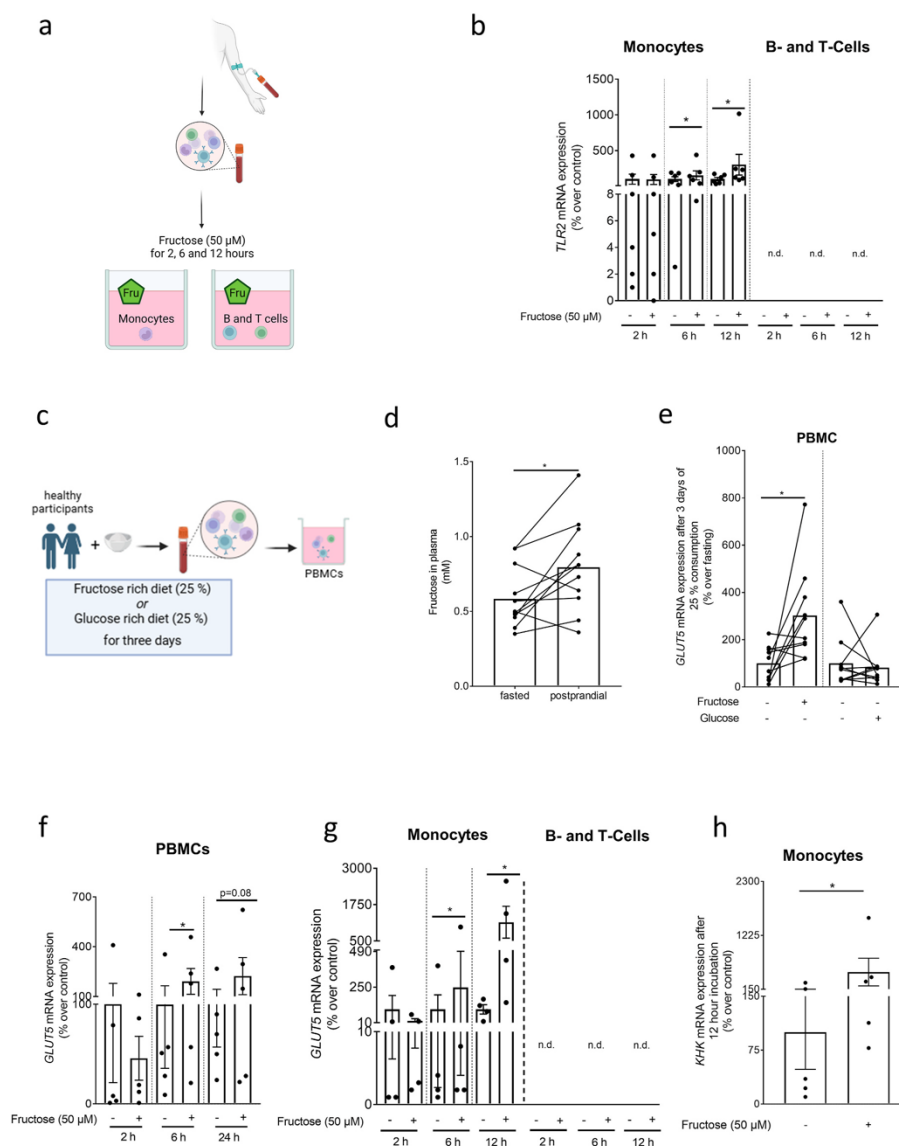


Fig. 1. Expression of *TLR2*, *GLUT5*, and *KHK* mRNA in PBMCs, monocytes, and B- as well as T-cells isolated from healthy donors or healthy humans after the intake of fructose. (a) Schematic drawing of experimental set-up of experiments shown in (b) and (f, g, h). (b) *TLR2* mRNA expression in monocytes and B- and T-lymphocytes isolated from buffy coats and *ex vivo* challenged with fructose (50 μ M) for 2, 6 and 12 h. (c) Study design of the human intervention study (study A) with an acute intake of fructose (40 g) and a fructose rich diet for 3 days (25 % E of total caloric intake). (d) Fructose levels in plasma before and 3 h after a light breakfast containing 40 g fructose as well as (e) *GLUT5* mRNA expression in isolated PBMCs after three days of a fructose or glucose rich diet (25 % E of total caloric intake) of healthy participants. (f) *GLUT5* mRNA expression in PBMCs from healthy human donors challenged *ex vivo* with fructose (50 μ M) for 2, 6 and 24 h. (g) *GLUT5* mRNA expression in monocytes and B- and T-cells of healthy human donor's *ex vivo* challenged with fructose (50 μ M) for 2, 6 and 12 h. (h) *KHK* mRNA expression in monocytes isolated from buffy coats and challenged with fructose (50 μ M) for 12 h * $p \leq 0.05$. Fig. 1 (a); n = 6; Fig. (d, e): n = 10; Fig. (f): n = 5; Fig. (g): n = 4; Figure (h): n = 5. Fig. 1 (a) and (c) were created with biorender.com. TLR2: Toll like receptor 2; GLUT5: glucose transporter 5; PBMC: peripheral blood mononuclear cells; KHK: ketohexokinase.

Table 1

Expression of *TLR1* and 6 in human monocytes isolated from buffy coats and incubated $\pm 50 \mu\text{M}$ fructose for 2, 6 or 12 h.

Parameter	2 h		6 h		12 h	
	C	Fructose (50 μM)	C	Fructose (50 μM)	C	Fructose (50 μM)
<i>TLR1</i> mRNA expression [#]	100	114.7 $\pm$ 35.1	100	113.8 $\pm$ 28.9	100	64.9 $\pm$ 11.9
<i>TLR6</i> mRNA expression [#]	100	81.0 $\pm$ 31.2	100	89.0 $\pm$ 21.1	100	90.0 $\pm$ 29.7

Values are means $\pm$ standard error of means. [#] % over control; n = 6–9; C: untreated cells, control; TLR: Toll like receptor.

induced after 6 and 12 h when compared to naïve controls (Fig. 1g). Moreover, mRNA expression of ketohexokinase (*KHK*) being the pace-maker enzyme of fructose metabolism [20], was also significantly induced by ~ 5 -fold in monocytes exposed to fructose for 12 h (Fig. 1h).

3.2. Effect of fructose on LTA-dependent stimulation of monocytes

To determine if the exposure to fructose also affects the immune response of monocytes to lipoteichoic acid (LTA), the latter being derived from the walls of *Staphylococcus aureus* and has been shown before to activate immune cells through TLR2-dependent signaling cascades [21], monocytes pre-exposed to a fructose enriched cell culture media were challenged with 10 $\mu\text{g}/\text{ml}$ LTA (Fig. 2a for experimental set-up). Expressions of tumor necrosis factor alpha (*TNF α*), interleukin-6 (*IL-6*), and interleukin-1 β (*IL-1 β*) mRNA were significantly enhanced by ~ 3.2 -, ~ 2.7 -, and ~ 5.8 -fold in LTA-treated cells pre-exposed to fructose compared to cells only treated with LTA. Exposure to fructose alone had no effects on the mRNA expression of the three cytokines when compared to naïve cells (Fig. 2b–d). Moreover, when exposing differentiated Caco-2 cells grown in a *trans*-well system for 14 h to fructose

(10 mM) on the apical side of the *trans*-well system, GLUT5 protein levels in Caco-2 cells were significant increased by ~ 1.2 -fold while in monocytes cultivated in the basolateral compartment of the *trans*-well system, mRNA expression of *GLUT5* was induced by ~ 8 -fold compared to controls (Fig. 3, b + c). Fructose levels in the basolateral compartment of the co-culture model increased from 0 μM to $\sim 250 \mu\text{M}$ (~ 2.5 % of the fructose concentration added to the apical compartment, Fig. 3d). The addition of LTA for 2 h to the basolateral compartment of *trans*-wells exposed to fructose resulted in a significantly higher expression of *TNF α* mRNA (~ 1.9 -fold) in monocytes when compared to monocytes cultivated underneath Caco-2 cells treated with plain cell culture media (Fig. 3e).

3.3. Effect of fructose and maltodextrin intake in healthy human subjects on the LTA-dependent induction of proinflammatory cytokines in monocytes

To determine if fructose also alters immune responsiveness of monocytes after an acute ingestion of fructose in humans, healthy subjects were asked to either consume 1 L of a fructose or a maltodextrin

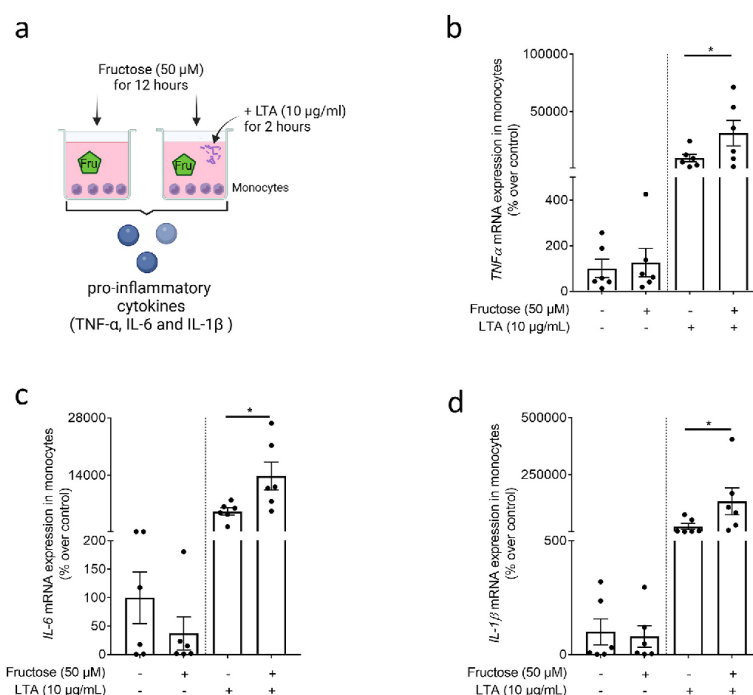


Fig. 2. Effect of fructose on the LTA-dependent induction of proinflammatory cytokine expression in monocytes obtained from healthy donors. (a) Graphical illustration of experimental set-up. (b) *TNF α* , (c) *IL-6* and (d) *IL-1 β* mRNA expression in monocytes pre-incubated for 12 h with 50 μM fructose and challenged with 10 $\mu\text{g}/\text{ml}$ LTA for 2 h n = 6. * $p \leq 0.05$. Fig. 2 (a) was created with [biorender.com](https://www.biorender.com). LTA: lipoteichoic acid; TNF α : tumor necrosis factor; IL: interleukin.

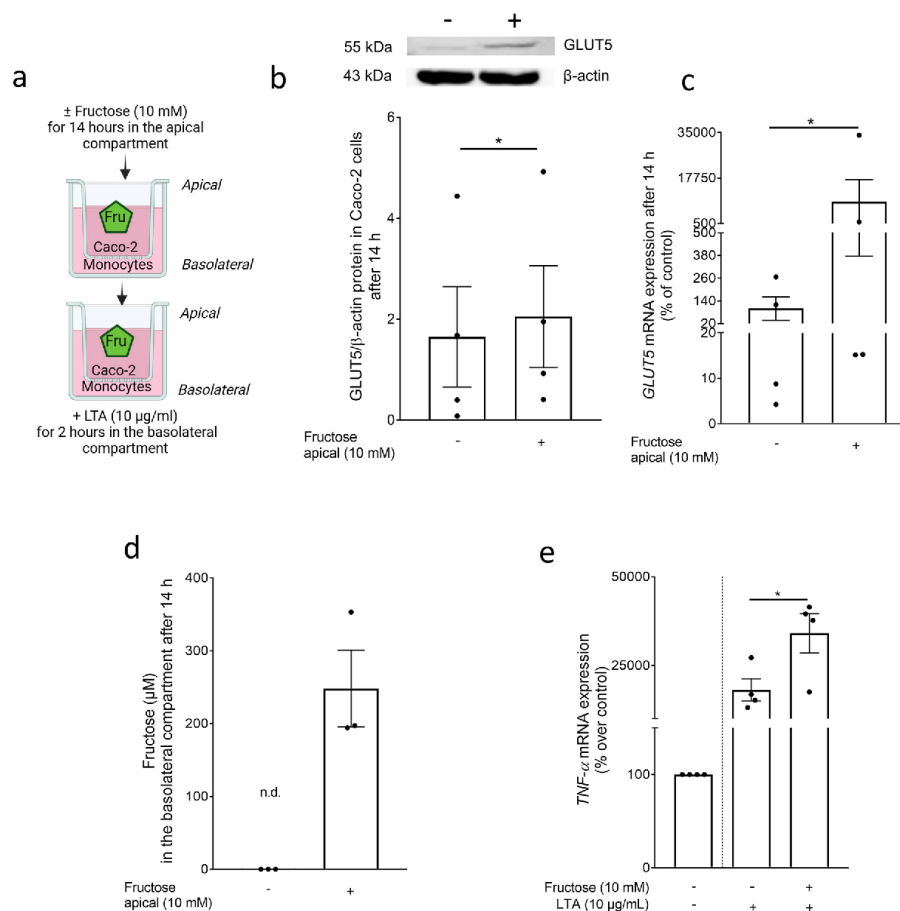


Fig. 3. Effect of fructose on fructose transport and the LTA-dependent induction of *TNFα* mRNA expression in a co-culture model of differentiated Caco-2 cells and human primary monocytes. (a) Experimental set-up. (b) GLUT5 protein concentration in Caco-2 cells and (c) *GLUT5* mRNA expression in monocytes 14 h after challenging Caco-2 cells with 0–10 mM fructose (d) fructose concentration in the basolateral compartment as well as (e) *TNFα* mRNA expression in monocytes after the 14 h challenge of Caco-2 cells with fructose (10 mM) and 2 h incubation of monocytes with LTA (10 µg/ml). n = 3–4. **p* ≤ 0.05. Fig. 3 (a) was created with biorender.com. GLUT5: glucose transporter 5; LTA: lipoteichoic acid; TNFα: tumor necrosis factor.

sweetened beverage containing 110 g of either sugar after being standardized in their dietary intake for 2 days (for study design and baseline characteristics see Fig. 4a and Supplemental Table S2). The intake of the fructose enriched beverage was again related with a significant increase in plasma fructose levels (Fig. 4b). Moreover, upon stimulation with LTA, the relative release (compared to unstimulated) of TNFα and IL-6 proteins from monocytes isolated after fructose ingestion was significantly higher by ~1.4- and ~1.5-fold, respectively, compared to that found after subjects consumed maltodextrin. After the fructose intake, IL-1β protein levels in the cell culture media of monocytes challenged with LTA were ~1.5-fold higher (*p* = 0.06) than that detected after the intake of maltodextrin (Fig. 4c–e).

3.4. Effect of a SP1 inhibitor mithramycin and a ketohexokinase inhibitor on the fructose- and LTA-related induction of TLR2 and proinflammatory cytokines in monocytes

As studies suggest that the transcription factor SP1 may be critical in the regulation of TLR2 and fructose has been suggested to alter SP1 activity [22], we next determined if SP1 is critical in the fructose-dependent induction of TLR2 in monocytes (Fig. 5). SP1 protein levels were significantly higher in cell homogenates of fructose-challenged monocytes compared to control cells (Fig. 5a). The induction of SP1 was related with a significant decrease of ATP levels in cells challenged with fructose for 2 h which was no longer detectable 6 and 12 h after the fructose challenge was initiated (Fig. 5b). Furthermore, pre-treating isolated monocytes with a SP1 inhibitor (mithramycin) and a KHK inhibitor (KHK inhibitor 1), respectively, blunted the fructose-dependent induction of *TLR2* mRNA expression almost completely to the level of controls (Fig. 5 c + d). Moreover, *SP1* mRNA

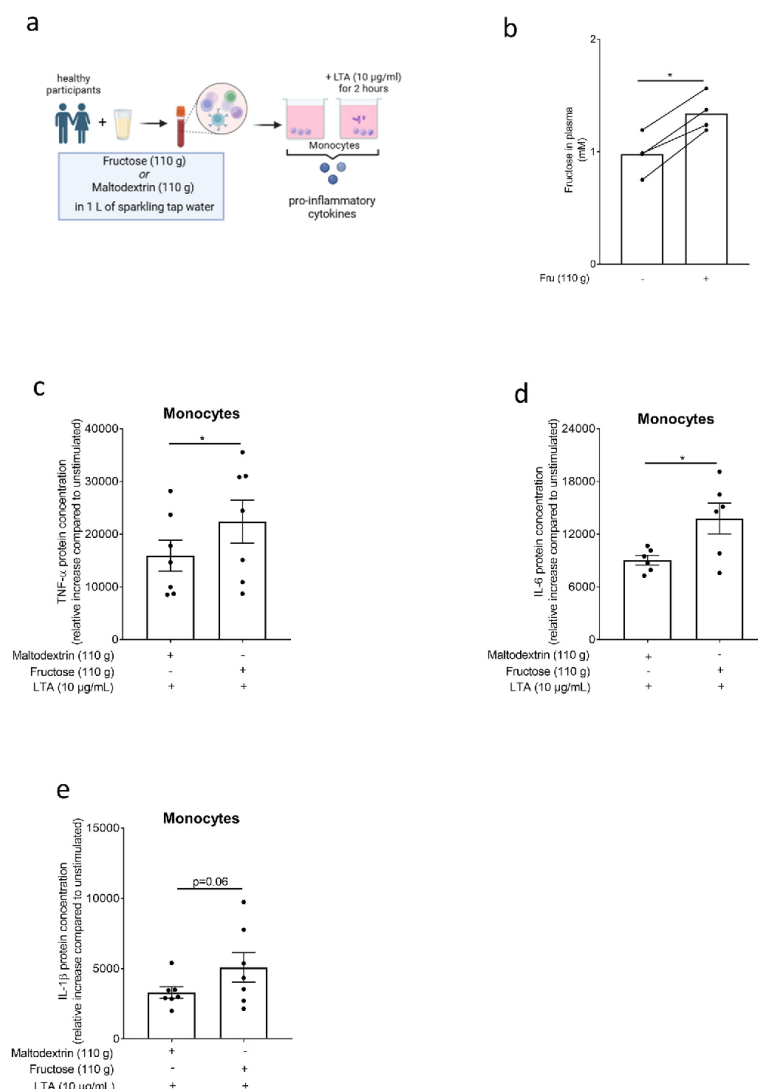


Fig. 4. Effect of an acute intake of an isocaloric fructose- or maltodextrin-rich beverage on fructose levels in blood and LTA-induced cytokine release in monocytes. (a) Study design of the human intervention study (study B) and ex vivo experiments. (b) Plasma fructose levels 2 h after the intake of 110 g fructose. Relative increase (compared to unstimulated) of (c) TNF-α, (d) IL-6, and (e) IL-1β protein concentration in monocytes isolated after the intake of maltodextrin or fructose challenged ex vivo with and without 10 µg/mL LTA for 2 h n 4–7. * $p \leq 0.05$. Fig. 4 (a) was created with biorender.com.; LTA: lipoteichoic acid; TNF-α: tumor necrosis factor; IL: interleukin.

expression was significantly lower in fructose-treated monocytes concomitantly treated with KHK inhibitor 1 compared to fructose + vehicle-treated cells (Fig. 5e). After incubating fructose-treated monocytes with KHK inhibitor 1 for 12 h and a challenge with LTA for further 2 h the mRNA expression of IL-1β was significantly lower compared to monocytes without the inhibitor (Fig. 5f).

4. Discussion

Studies suggested that especially in industrialized countries nutritional intake and dietary pattern may not only be related to the development of metabolic diseases but may also increase the susceptibility to (bacterial) infectious diseases [23]. While intense research efforts have been attributed to unravel molecular mechanisms underlying bacterial infections and to develop new treatment measures besides antibiotics, the understanding of the role of nutrition and specific nutrients in the

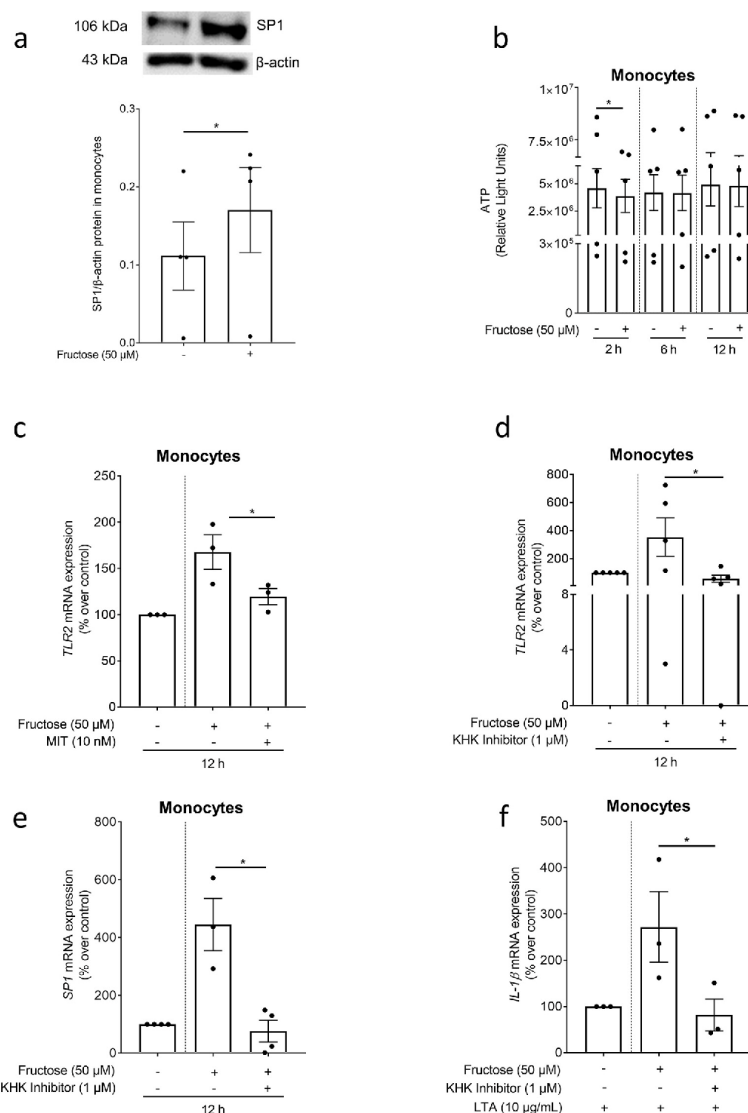


Fig. 5. Effect of fructose on SP1 protein and ATP levels in primary monocytes and on SP1 and KHK-1-dependent regulation of TLR2 expression in primary monocytes. Effect of the incubation of monocytes with fructose (0–50 μ M) for 12 h on (a) SP1 protein concentration. (b) ATP units in monocytes after 2, 6 and 12 h of incubation with fructose. TLR2 mRNA expression in monocytes incubated with fructose (c) $\pm$ MIT (10 nM) and (d) $\pm$ KHK inhibitor 1. (e) SP1 mRNA expression of monocytes incubated with fructose $\pm$ KHK inhibitor 1 (1 μ M) and (f) IL-1 β mRNA expression after additional stimulation with LTA (10 μ g/mL) for 2 h n = 3–5. $^*p \leq 0.05$. MIT: mithramycin; SP1: transcription factor 1; TLR2: Toll like receptor 2; KHK: ketohexokinase.

development and progression of bacterial infections is still limited. Employing short-term human intervention studies and *ex vivo* stimulation experiments in primary human immune cells we here report that the intake of free fructose is related with an induction of TLR2 expression in monocytes and subsequently an increased responsiveness of these cells to the Gram-positive bacteria derived toxin LTA. The amounts of fructose employed in the present acute and short-term intervention study may seem rather high (44–110 g, for comparison, 1 L of commercially

cafein-free soft drink on average contains 90 g of sucrose (sucrose in Europe) translating into 45 g of fructose). Studies conducted in industrialized countries suggest that especially in adolescents and young adults these amounts of fructose can be reached on a daily basis when sugar sweetened beverages (e.g., soft drinks and yoghurt drinks) and sweets (e.g., chocolates, chewy sweets, and confectioneries) are included in the diet [24]. In the present study we found that an acute intake of this amount of free fructose resulted in a significant increase of

fructose levels in peripheral blood within 2 h after ingestion being related to a significant increase in *GLUT5* and *KHK* mRNA expression in monocytes. Effects alike were not found in B- and T-cells. Moreover, we found a marked induction of *GLUT5* mRNA expression in primary human monocytes cultivated below Caco-2 cells grown on *trans*-well inserts upon exposure of Caco-2 cells to moderate amounts of fructose. Interestingly, while Caco-2 cells were exposed to 10 mM fructose apical, after 14 h, fructose concentration in the basolateral compartment was only ~250 μ M suggesting that either Caco-2 cells and/or monocytes rather efficiently metabolize fructose. It has been shown before by others that the oral ingestion of fructose is related with increased levels of the monosaccharide in peripheral blood similar to those found in the present study [25–27]. Also, others reported before that in enterocytes *GLUT5* mRNA expression is rather rapidly induced upon exposure to fructose and that when challenged with 'normal' doses of the monosaccharide the greater proportion of it is metabolized into glucose and triglycerides, while only limited amounts are being released as fructose into the circulation at the basolateral side of the cells [28,29]. Results of *in vitro* studies suggest that *GLUT5* almost exclusively functions as D-fructose [30] transporter. In line with our findings, results of others also suggested before, that monocytes metabolize fructose [31].

Somewhat contrasting the findings of the present study, it has been reported by others that a chronic exposure to high amounts of fructose may alter B- and T-cell development and function in rodents [32,33]. Also, *in vitro* studies suggest that B-cells show mitochondrial oxidation of fructose when the sugar is administrated alone [34] whereas studies in mice suggest that fructose-related effects on T-cells e.g., in priming antitumor CD8⁺ T-cells or modulating T-cells population, are related to indirect effects involving other cells or intestinal microbiota [35,36]. In line with our findings, Jones et al., also reported no direct effects of fructose on cell proliferation when challenging CD4⁺ and CD8⁺ T-cells [31]. However, to the best of our knowledge systematic studies assessing the direct effect of fructose on B- and T-cell function, respectively, in humans are still lacking. Taken together, results of our study suggest that the intake of free fructose in concentrations that are achievable through a 'normal' diet can result in an induction in *GLUT5* and *KHK* expression in monocytes in healthy humans. Our results further suggest that at least upon a short-term exposure, similar effects are not found in other blood immune cells e.g., B- und T-cells and that enterocytes metabolize marked amounts of fructose upon exposure. Further studies are needed to determine if a chronic intake of elevated amounts of free fructose also alters fructose uptake and metabolism in monocytes.

Results of previous studies of our group suggested that an increase in dietary free fructose but not in free glucose intake is related with an increased expression of *TLR2* mRNA expression in PBMcs [16]. In line with these findings the challenge of monocytes isolated from buffy coats of healthy donors with fructose was related with an induction of *TLR2* mRNA while *TLR1* and 6 mRNA expression remained unchanged. Similar effects were not found for glucose and in B- or T-cells. The latter finding is somewhat in line with the findings of Jones et al., who found no direct effects of fructose in T-cells [31]. Moreover, the induction of *TLR2* was related with an enhancement of proinflammatory cytokine release when cells were challenged with LTA both when monocytes were directly exposed to the monosaccharide or when being grown beneath Caco-2 cells challenged with fructose. In line with these findings the release of cytokines following the intake of fructose and *ex vivo* stimulation of cells with LTA was also significantly higher than when subjects had consumed maltodextrin (=glucose). It has been shown before that a challenge of monocytes with fructose can alter their metabolic profile and that fructose may alter their phenotype towards a more inflammatory [31] one. Moreover, in the same study, the concomitant feeding of mice with glucose and fructose resulted in increased serum levels of proinflammatory cytokines upon a challenge with lipopolysaccharide and alterations of fructose metabolism [31]. In studies of others the enhanced release of proinflammatory cytokines following the challenge with fructose and Gram-negative bacteria derived endotoxin was

primarily related to a fructose-dependent increase in mTORC1 activity [37,38]. In the present study, we found that the induction of *TLR2* expression and subsequent higher responsiveness of monocytes to a challenge with LTA was dependent upon the metabolism of fructose and related decrease in ATP levels as well as induction of SP1. Indeed, the concomitant treatment with a KHK inhibitor or a SP1 inhibitor attenuated the induction of *TLR2* and enhanced the release of proinflammatory cytokines of monocytes when being challenged with LTA. It has been shown by others before that fructose metabolism may acutely deplete cellular ATP levels [39]. Moreover, SP1 has been suggested to be one of the transcription factors involved in the regulation of *TLR2* mRNA expression in humans [22]. It further has been shown that fructose may alter activity of protein phosphatase 2 (PP2A) through cellular ATP levels which in turn has been shown to also alter phosphorylation of SP1 [40]. However, our data also suggest that *SP1* mRNA expression is regulated through mechanisms related to fructose metabolism.

Taken together, results of the present study suggest that the ingestion of fructose may enhance LTA-dependent immune responsiveness in healthy humans through a SP1-dependent induction of *TLR2* expression. Results of the present study by no means preclude that fructose may not also affect responsiveness to bacterial or viral challenges by other means; however, our data suggest that in human monocytes the metabolites derived through the metabolism of fructose may alter *SP1* and subsequently *TLR2* mRNA expression. Further studies are needed to assess if similar alterations are also found when fructose is consumed at elevated levels for an extended period of time or if fructose is consumed in different matrices.

Our intervention studies are not without limitations that need to be considered when interpreting the data. A key limitation is that in both intervention studies only young healthy adults were enrolled. Results may be different in children or elderly as well as in overweight or metabolically diseased subjects. This will have to be assessed in future studies. Also, we only assessed the acute effects of fructose *ex vivo*. It remains to be determined if effects alike are found when fructose is consumed regularly and in settings of 'real' infections with Gram-positive bacteria. However, due to ethical reasons studies assessing the effects of fructose in the course of infections *in vivo* would need to focus on animal studies. At least in rodents it has been shown that the regulation of *TLR2* in immune cells may differ from that in humans [22]. Also, if effects on *TLR2* expression and signaling differ if fructose is consumed in a natural matrix e.g., as part of fruit (juice) or vegetable remains to be determined, too.

5. Conclusion

In summary, results of the present study suggest that an acute elevated intake of fructose may enhance the *TLR2*-dependent response to LTA in blood monocytes in healthy humans thereby bolstering the hypothesis that certain nutritional factors like sugar may alter immune responsiveness of humans. Our results further suggest that this enhancement of the LTA-dependent immune response is mediated through an activation of the transcription factor SP1. Moreover, results of the present study also suggest that herein the intracellular metabolism of fructose is critical. However, if and how signaling pathways shown in the present study affect disease outcomes in setting of Gram-positive infections in humans and if adaptive responses e.g., a regular consumption of fructose or the matrix in which the sugar is consumed alter the effects of fructose on *TLR2* signaling remains to be determined.

CRedit authorship contribution statement

Raphaela Staltner: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis. **Katja Csarman:** Writing – review & editing, Investigation. **Amelie Geyer:** Writing – review & editing, Investigation. **Anika Nier:** Writing – review & editing, Investigation. **Anja Baumann:** Writing – review & editing,

Investigation. Ina Bergheim: Writing – review & editing, Writing – original draft, Supervision, Conceptualization.

Declaration of competing interest

All authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2025.103729>.

Data availability

Data will be made available on request.

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2.3. Publication 3: A single oral dose of an iso-alpha acids rich hop extract dampens the lipoteichoic acid mediated immune response of monocytes in healthy individuals

Katja Csarmann, Finn Jung, Anja Baumann, Benedikt Simbrunner, Kerstin Schweiger, Katharina Burger, Raphaela Staltner, Claus Hellerbrand, Ina Bergheim. Eur J Nutr. 2026 Mar; 65(3):84. doi: 10.1007/s00394-026-03931-x

Highlights:

- An oral dose of 15 mg IAA-rich extract reduced LTA-induced IL-6 release in monocytes within one hour.
- PBMCs from IAA-treated participants showed significantly lower IL-6 and IL-1 β secretion after LTA stimulation compared to placebo.
- IAA suppressed JNK-mediated inflammatory signalling without affecting TLR2 activation.

Contribution to the manuscript:

- Planning parts of the study and preparing the ethics application
- Conducting parts of the human intervention study
- Conducting the *ex vivo* stimulation experiments
- Performing laboratory chemical analyses of human samples
- Analysing the data and performing statistical evaluations
- Preparing tables and figures and drafting the manuscript

Total contribution: 30%



A single oral dose of an iso-alpha acids rich hop extract dampens the lipoteichoic acid mediated immune response of monocytes in healthy individuals

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Abstract

Purpose Bacterial infections significantly contribute to global mortality. Lipoteichoic acid (LTA), a key component of Gram-positive bacterial cell walls, triggers immune responses via Toll-like receptor 2 (TLR2). Iso-alpha acids (IAA), bitter compounds derived from hops (*Humulus lupulus L.*), are known for their anti-inflammatory properties, but their effects on human immune modulation remain unclear. This study explored the effect of a single oral dose of IAA on LTA-induced inflammatory responses in human immune cells and underlying molecular mechanisms.

Methods In a pilot study in healthy female volunteers ($n=5$) dose- and time-dependent effects (0–90 mg IAA) on LTA-induced immune responses in monocytes were assessed. A randomized, placebo-controlled cross-over study ($n=13$, male and female) evaluated the acute effects of 15 mg IAA. Peripheral blood mononuclear cells (PBMCs) were stimulated with LTA ex vivo, and cytokine release (IL-6, IL-1 β) was measured. Mechanistic studies using J774A.1 cells and TLR2-transfected HEK293 cells explored the molecular pathways of IAA.

Results In the pilot study, 15 mg IAA was identified as the most tolerated dose in terms of taste for the participants which still had a marked effect on LTA-mediated IL-6 release from monocytes. In the main study, PBMCs from IAA-treated participants showed significantly lower IL-6 and IL-1 β secretion after LTA stimulation compared to placebo ($p<0.05$). In vitro, IAA suppressed JNK-mediated inflammatory signaling without affecting TLR2 activation.

Conclusion A single low-dose intake of IAA from hops reduces LTA-induced cytokine release in blood immune cells, likely via JNK inhibition, suggesting its potential role in modulating inflammatory responses.

C. Hellerbrand: Deceased.

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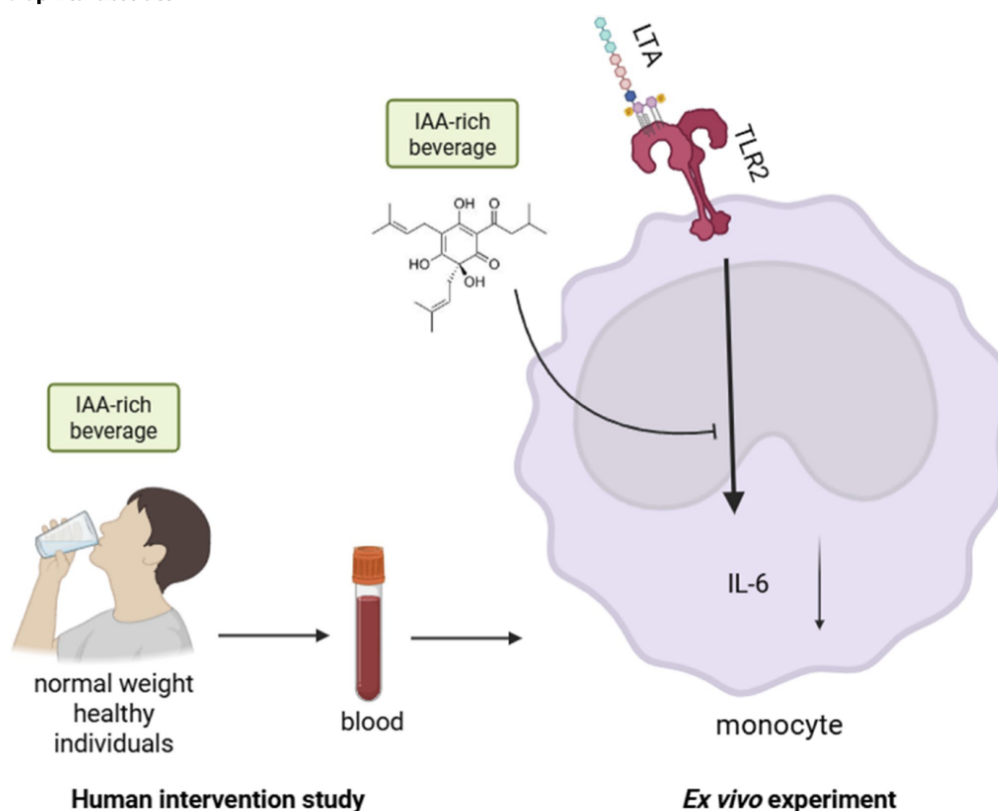
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Graphical abstract



Keywords Iso-alpha acids · Lipoteichoic acid · Monocytes · Toll-like receptor 2 · Cytokines

Abbreviations

ALT	Alanine aminotransferase
AMR	Antimicrobial resistance
ANOVA	Analysis of variance
AP-1	Activator protein 1
AST	Aspartate aminotransferase
BMI	Body mass index
CD14	Cluster of differentiation 14
ELISA	Enzyme-linked immunosorbent assay
HEK	Human embryonic kidney (cells)
HRP	Horseradish peroxidase
IAA	Iso-alpha acids
IKK β	I κ B kinase beta
IL	Interleukin
JNK	c-Jun-N-terminal kinase
LTA	Lipoteichoic acid

MAPK	Mitogen-activated protein kinase
NF κ B	Nuclear Factor kappa light chain enhancer of activated B cells
PBMC	Peripheral blood mononuclear cells
RIPA buffer	Radio-immunoprecipitation assay buffer
RPMI	Roswell park memorial institute (medium)
SEM	Standard error of the mean
TLR	Toll-like receptor

Introduction

Bacterial infections still cause a significant number of deaths worldwide every year [1], and antibacterial resistance (AMR) marks a continuous challenge in the management of infections. For instance, it has been estimated that

in 2021 ~ 1.14 million deaths worldwide were attributable to bacterial AMR [2]. Results of the Global Burden Disease 2021 Antimicrobial Resistance Collaborators suggest that AMR deaths in children under the age of 5 years decreased in the last decades [2], whereas the number of deaths related to AMR in those older than 70 years being among the fastest growing population world-wide, is steadily increasing [2]. Gram-positive bacteria like *Staphylococcus aureus*, *Streptococcus pneumoniae* and a variety of bacteria summarized under the so-called vancomycin-resistant *Enterococci*, have been shown to have a highly variable growth and resistance pattern. These bacteria are also among the leading bacterial cause of death in over 15-year-old individuals in 135 countries [1, 3]. Studies suggest that not only the bacteria and their growth but also surface structures which in the case of Gram-positive bacteria include lipoproteins, peptidoglycans, poly-N-acetyl-glucosamine, wall teichoic acid and lipoteichoic acid (LTA) are critical in the inflammatory response related to bacteria [4–6]. LTA is indispensable in *Staphylococcus aureus* [7] and has been shown to induce inflammatory responses in the host through Toll-like receptor 2 (TLR2), a pattern recognition receptor (PRR) that either forms homodimers or heterodimers with TLR1 or TLR6 [8]. Previous studies suggest that similar to the recognition of bacterial endotoxin by TLR4 [9, 10], CD14 can bind LTA and transfer the bacterial wall compound to TLR2 [11, 12] subsequently leading to an induction of proinflammatory signaling cascades and a release of respective cytokines [13–15].

For many centuries hop (*Humulus lupulus* L.) has been used in beer brewing and in traditional medicine for the treatment of insomnia but also inflammatory diseases [16, 17]. Indeed, hop constituents are added to the brewing process for their taste but also their antimicrobial properties [18]. In vitro studies have suggested that bitter acids like alpha-acids such as humulone found in hop possess antimicrobial properties against Gram-positive bacteria including *Staphylococcus*, *Clostridium* and *Bacillus* [19–23]. Moreover, results of animal studies suggest that the oral intake of iso-alpha acids (IAA) derived from alpha acids upon thermal isomerization [24] can attenuate inflammatory responses like the induction of proinflammatory cytokines or oxidative stress in liver tissue and brain in models of alcohol- and diet-related liver damage and Alzheimer's disease [13, 25, 26]. Results of in vitro studies suggest that IAA may alter NFκB and AP-1 activation through interfering with JNK and IKK-beta activation [25, 27]. Pharmacokinetic studies in humans have shown that an intake of ~ 600–800 ml of commercially available high- and low hopped beer (Pale Ale: ~40 mg/l and wheat beer: ~10 mg/l IAA) leads to blood trans-IAA concentrations of ~ 0.1 and 0.02 mg/l, respectively, peaking at 0.5 h and dropping to ~ 0.01–0.02 mg/l and 0.003 mg/l

after 2 h [28]. It has also been reported that IAA was measurable up to 6 h after consumption. These data suggest that IAA is absorbed, bioavailable and may exert its physiological effects over a prolonged period after ingestion [28, 29]. If iso-alpha acids when consumed in doses corresponding to those found when consuming moderate amounts of alcohol-free or alcohol containing beer, have effects on the immune response in humans has not yet been clarified.

Starting from this background, the aim of this study was first to determine in a pilot study if the oral intake of low doses of an IAA-rich hop extract (0–90 mg IAA) at concentrations also reached in the brewing process of alcohol containing or alcohol-free beer is related with alterations of the immune response of blood derived monocytes and how long these effects are prevalent in young healthy women. In a second randomized placebo-controlled crossover study, we assessed the effect of a onetime ingestion of an IAA-rich hop extract (15 mg IAA) or placebo in a mixed-sex cohort of young healthy individuals and determined related molecular mechanisms.

Materials and methods

Human intervention studies

All intervention studies were carried out in a randomized placebo-controlled cross-over design and in accordance with the ethical standards laid down in the Declaration of Helsinki of 1975 as revised in 1983. Studies were approved by the ethics committee of the University of Vienna, Vienna, Austria (reference numbers: 00367 and 00848) and were registered at clinical trials (NCT04847193, 16.03.2021; NCT06286644, 17.01.2024). The following exclusion criteria were defined for both studies: (1) following a special diet or (2) food malabsorption or (3) a history of diseases of the gastrointestinal tract or (4) the use of anti-inflammatory medication, whereas the following inclusion criteria were defined: normal weight (BMI > 18.5 kg/m² or < 24.9 kg/m²), non-smoking people were enrolled after giving written informed consent and they should not have a viral or bacterial infection 3 weeks prior the study. In both studies, the participants were asked to refrain from hop-containing products two weeks prior to the studies and during the intervention.

Pilot study to determine time- and dose-response

To determine the optimal time of cell isolation after the ingestion of the IAA-rich hop extract and the optimal IAA doses, a single blinded, randomized pilot study was conducted in normal weight, healthy female volunteers. To

prepare the study drink, which was prepared freshly right before the intake, 10 ml drinking water were mixed with thickener (Nestlé S.A., Vevey, Switzerland), sugar-free lemon flavour (SodaStream GmbH, Frankfurt am Main, Germany) and 0, 15, 45 or 90 mg IAA derived through an IAA-rich hop extract (Isohop®, generous gift from Barth-Haas Group GmbH & Co. KG Nuremberg, Germany). After an overnight fast and obtaining a blood sample, subjects were asked to consume the study drink within 15 min together with a standardised breakfast (2 medium-sized pretzels with 30 g butter). Blood was taken at 1, 2, 3, 4 and 6 h after consumption of the study drink. Between time point 4 and 6 there was another standardised meal. After the first study day and a 1-week wash-out phase, in which the participants again were asked to refrain from all hops containing foods and beverages, the intervention was repeated in a cross-over design until all four study days were completed. The study design and the procedure on the study day are summarized in Fig. 1a. Clinical parameters e.g. fasting glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and cholesterol were determined in the serum and monocytes were isolated from the whole blood as detailed below.

Assessment of the acute effects of the IAA-rich hop extract

In a second study which was based on the results of the time- and dose-response study, the effect of 15 mg IAA derived through an IAA-rich hop extract compared to a placebo were assessed. Male and female participants were randomly assigned in a single-blinded design to receive a study drink as described before, containing 0 or 15 mg of IAA derived through an IAA-rich hop extract. The study drink was consumed within 15 min together with a standardised breakfast (2 medium-sized pretzels with 30 g butter). The participants were fasted overnight and blood samples were taken before the study drink was consumed and 1 h after consumption of the study drink. After the first study day and a 1-week wash-out phase, in which the participants were again asked to refrain from hops-containing foods and beverages, the intervention was repeated in a cross-over design. The study design and the procedure on the study day is summarized in Fig. 2a. Clinical parameters were determined in the serum and PBMCs were isolated from whole blood as detailed below. Demographic characteristics of the study subjects are reported in Tables 1 and 2.

Isolation of monocytes

Monocytes were isolated from whole blood samples collected from participants of the time- and dose-response study or from healthy, naïve, normal weight donors. After obtaining written informed consent and an overnight fast,

blood was drawn from healthy, normal weight donors, with approval from the ethics committees of the University of Vienna, Vienna, Austria (reference number 00586). Monocytes were isolated from whole blood samples as detailed before using a density gradient centrifugation and the pluriSelect system following the instructions of the manufacturer (pluriSelect Life Science UG & Co. KG, Leipzig, Germany). In brief, blood was layered onto monocytes density gradient media (1.068 g/ml) in separation tubes (pluriSelect Life Science UG & Co. KG, Leipzig, Germany) and centrifuged at room temperature for 15 min. Cells were collected and used for further experiments as described below.

Isolation of PBMCs

PBMCs were isolated from whole blood samples as described in detail before [11]. In brief, cells were isolated by density gradient centrifugation. In brief, blood was layered onto Pancoll solution (1.077 g/ml) and centrifuged for 30 min at room temperature. Cells were collected and used for further experiments as described below.

Stimulation of monocytes and PBMCs obtained after an oral ingestion of iso-alpha acids derived through an IAA-rich hop extract

Isolated monocytes and PBMCs were cultivated in RPMI-1640 medium (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) with 10% fetal bovine serum (Pan-Biotech GmbH, Aidenbach, Germany) and 100 µg/ml streptomycin and 100 U/ml penicillin (Pan-Biotech GmbH, Aidenbach, Germany) at 37 °C in a humidified 5% CO₂ atmosphere for 1 h. Subsequently, cells were either challenged with 0–10 µg/ml LTA for 6 h in the pilot study and for 24 and 48 h in the assessment of the acute effects study, respectively. Cell culture supernatant was collected at the end of the experiment and stored at –80 °C until further use.

Experiments in monocytes isolated from naïve healthy donors

Isolated monocytes were cultivated in RPMI-1640 medium (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) with 10% fetal bovine serum (Pan-Biotech GmbH, Aidenbach, Germany) and 100 µg/ml streptomycin and 100 U/ml penicillin (Pan-Biotech GmbH, Aidenbach, Germany) at 37 °C in a humidified 5% CO₂ atmosphere for 1 h. Subsequently, cells were preincubated for 1 h with different concentrations of iso-alpha acids (0.3125–1.25 µg/ml) and then treated with 10 µg/ml LTA for 6 h. Cell culture supernatant was collected at the end of the experiment and stored at –80 °C until further use.

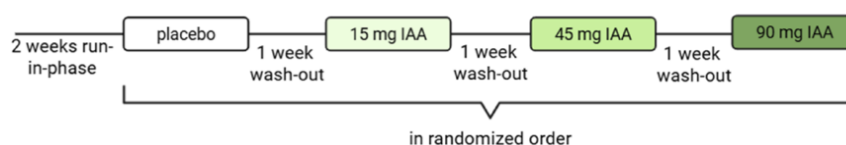
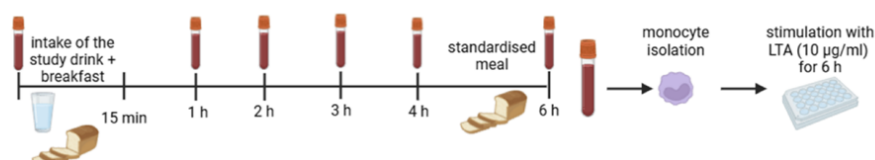
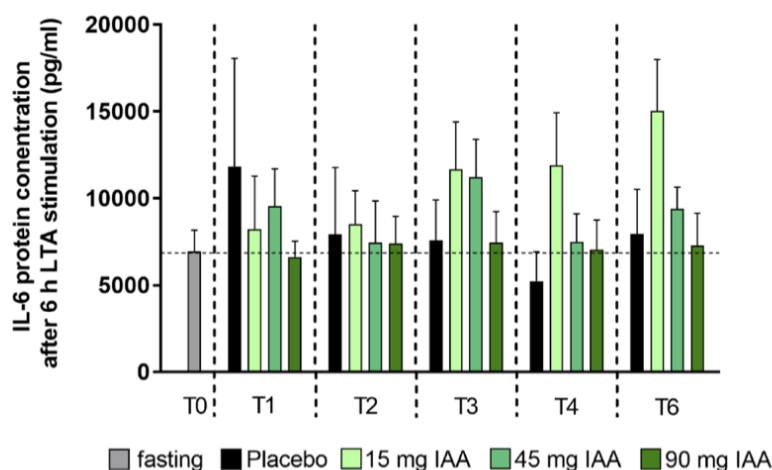
a**Study design:****Study day:****b**

Fig. 1 Effect of a single intake of different concentrations of an iso-alpha acids rich hop extract on the LTA-induced immune response of IL-6 protein concentration in monocytes of healthy study participants over time. **a** Study design and study day of the time- and dose-response study, **b** protein concentration of IL-6 in cell culture supernatant of monocytes stimulated with 10 µg/ml LTA for 6 h isolated from healthy female study participants of the time- and dose-response

study receiving either a placebo or IAA rich hop extract in different concentrations (0 mg, 15 mg, 45 mg or 90 mg). Data are presented as means ± SEM, $n=5$. The mean of IL-6 protein concentration of LTA-stimulated monocytes from fasting blood (T0) of all intervention groups are shown in one column. IAA: iso-alpha acids, IL: interleukin, LTA: lipoteichoic acid

Cell culture experiments with hTLR2 transfected HEK cells

A commercially available reporter gene assay with TLR2 transfected HEK293 cells was used to assess the effects of

iso-alpha acids on TLR2 ligand concentration (InvivoGen, Toulouse, France). The transfected human TLR2 cells were grown according to the instructions of the manufacturer. In brief, cells were grown in a humidified, 5% carbon dioxide atmosphere using DMEM media (Pan-Biotech GmbH,

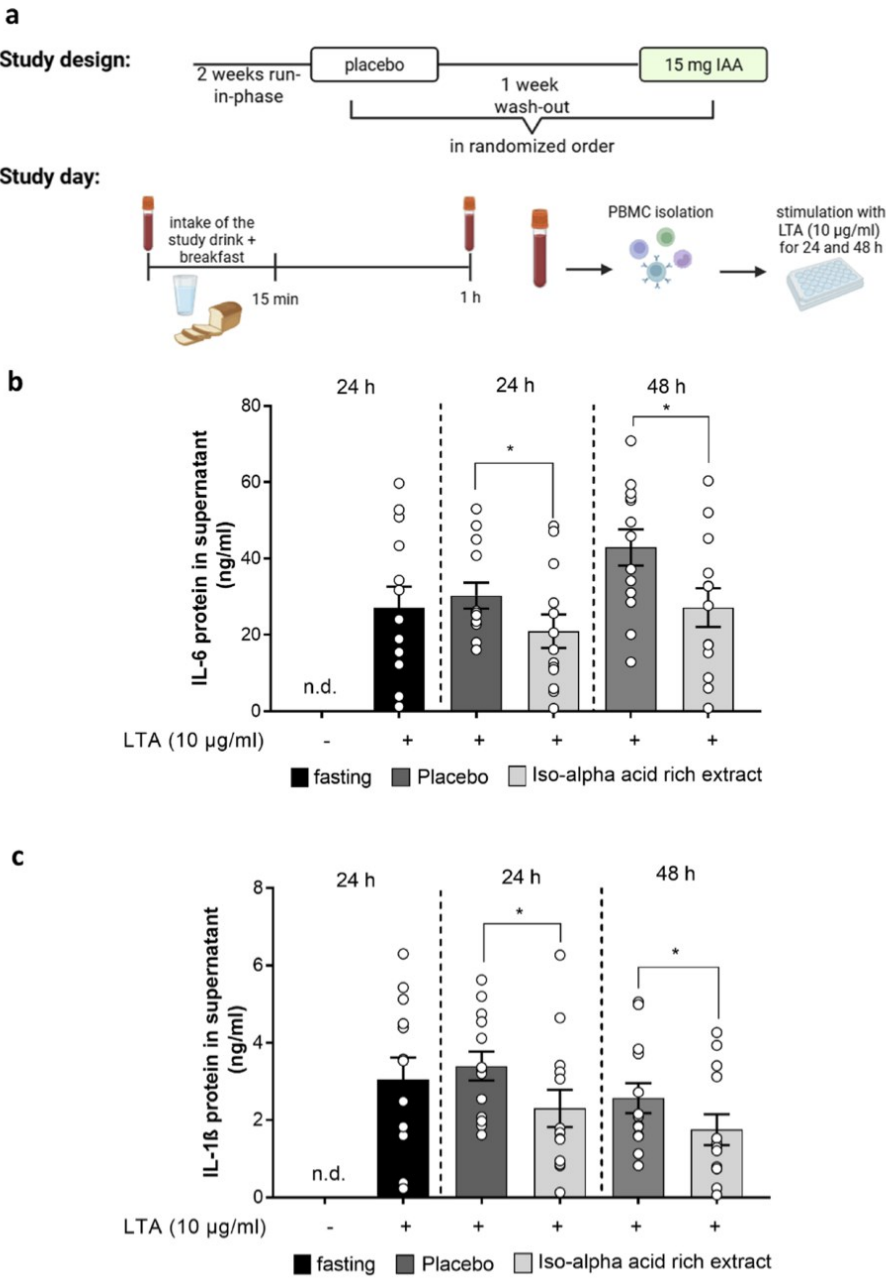


Fig. 2 Effect of a single intake of 15 mg of an iso-alpha acids rich hop extract on the LTA-induced immune response of proinflammatory cytokines in PBMCs of healthy study participants. **a** Study design and study day of the assessment of the acute effects of IAA, protein concentrations of **(b)** IL-6 and **(c)** IL-1 β in cell culture supernatant of PBMCs stimulated with 0–10 μ g/ml LTA for 24 h and 48 h isolated from healthy study participants of the assessment of the acute effects of IAA receiving either a placebo or 15 mg IAA rich hop extract. Data are presented as means $\pm$ SEM, $n=13$. * $p<0.05$. IAA: iso-alpha acids, IL: interleukin, LTA: lipoteichoic acid, n.d.: not detectable, PBMC: peripheral blood mononuclear cell

Aidenbach, Germany) containing 10% fetal bovine serum (Pan-Biotech GmbH, Aidenbach, Germany) and 100 μ g/ml streptomycin and 100 U/ml penicillin (Pan-Biotech GmbH, Aidenbach, Germany). After reaching 80% confluence, cells were challenged with 0–10 μ g/ml LTA in the presence of 0–40 μ g/ml iso-alpha acids dissolved in HEK-Blue™ Detection medium (InvivoGen, Toulouse, France) for 12 h. Colour changes of the medium being indicative of ligand concentration and binding were determined at 655 nm.

Cell culture experiments with J774A.1 cells

J774A.1 cells (DMSZ, Braunschweig, Germany) showing a morphology similar to macrophages/monocytes, were cultured in Dulbecco's Modified Eagle Medium (Pan-Biotech GmbH, Aidenbach, Germany) supplemented with 10% fetal bovine serum (Pan-Biotech GmbH, Aidenbach, Germany), 100 μ g/ml streptomycin and 100 U/ml penicillin (Pan-Biotech GmbH, Aidenbach, Germany) at 37 °C in a humidified 5% CO₂ atmosphere. At 80% confluency, cells were preincubated for 1 h with or without 5 ng/ml anisomycin (Biomol GmbH, Hamburg, Germany), afterwards they were preincubated for 1 h with iso-alpha acids (0–25 μ g/ml) and then treated with 10 μ g/ml LTA for 6 and 24 h. The supernatant was collected at the end of the experiment and stored at –80 °C until further use.

Enzyme-linked immunosorbent assays (ELISA's)

IL-6 and IL-1 β protein concentrations were analysed in cell culture supernatant of monocytes and PBMCs or J774A.1 cells using commercially available DuoSet® ELISA Development Systems (R&D®, Minneapolis, USA) kits.

Western blot analysis

J774A.1 cells were homogenized in RIPA buffer (20 mmol/l 3 [N-morpholino] propanesulfonic acid, 150 mmol/l NaCl, 1 mmol/l ethylenediaminetetraacetic acid, 1% Nonidet P-40, and 0.1% sodium dodecyl sulfate) containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) to obtain protein

lysates. Protein lysates (5 μ g/lane) were separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were further incubated with specific primary antibodies (phosphorylated c-Jun N-terminal kinase (JNK) and total JNK; phosphorylated p38 MAPK and p38 MAPK; Cell Signaling Technology, MA, USA) and the respective secondary antibody (anti-rabbit IgG, HRP-linked; Cell Signaling Technology, MA, USA). To detect the protein bands, Clarity Western Enhanced Chemiluminescence Substrate (Bio-Rad Laboratories, Hercules, CA, USA) and ChemiDoc XRS System (Bio-Rad Laboratories, Hercules, CA, USA) were used. Densitometric analyses of detected bands were performed with ImageLab (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analyses

Data are presented as means $\pm$ standard error of the means (SEMs). Grubb's test was performed before statistical analysis to identify outliers. Homogeneity of variances was tested and data were log-transformed if data were not normal distributed or in case of inhomogeneity of variances before performing further statistical tests. A paired t-test was used to determine statistically significant differences between interventions and to analyse differences between C- and LTA-stimulated cells of Western Blot analysis. One-way ANOVA was used for all other comparisons (GraphPad Prism Software, CA, USA). A p-value < 0.05 was defined as threshold for statistical significance.

Results

Effects of an acute oral intake of an IAA-rich hop extract on the LTA-induced IL-6 release in monocytes: assessment of time- and dose-response in a pilot study

Characteristics of the 5 study participants enrolled and analysed in the pilot study assessing the time- and dose-effect of an oral intake of IAA on the LTA-induced release of IL-6 are summarized in Table 1. IL-6 was selected as suitable target cytokine as it has been shown before in cell culture experiments that its bacterial toxin induced expression can be suppressed by IAA [13]. As expected, the challenge of monocytes isolated before and 1 h after the intake of the placebo resulted in a manifest ~ 1.85-fold increase in IL-6 protein levels in cell supernatant (see Supplemental Fig. 1). A similar effect of LTA was also found in cells isolated 2, 3, 4, and 6 h after the intake of the placebo (see Supplemental Fig. 1). In contrast, when monocytes isolated 1 h after the

Table 1 Characteristics of healthy female subjects enrolled in the dose- and time- response study

Parameter	Baseline
Sex (m/f)	0/5
Age (years)	26.4±1.7
Body weight (kg)	56.1±0.8
Height (m)	1.7±0.0
BMI (kg/m ²)	20.4±0.7
Fasting glucose (mg/dl)	86.0±3.4
AST (U/L)	22.6±3.4
ALT (U/L)	17.0±3.3
Cholesterol (mg/dl)	160.4±9.8
Triglycerides (mg/dl)	65.6±4.1

Values are means±standard error of means. ALT: alanine aminotransferase; AST: aspartate aminotransferase; BMI: body mass index

Table 2 Characteristics of healthy subjects enrolled in the study to assess the acute effect of IAA on LTA-dependent immune response of PBMCs

Parameter	Baseline
Sex (m/f)	6/7
Age (years)	26.5±0.9
Body weight (kg)	64.2±2.1
Height (m)	1.7±0.0
BMI (kg/m ²)	21.7±0.3
Fasting glucose (mg/dl)	88.2±1.9
AST (U/L)	23.5±1.6
ALT (U/L)	27.6±3.8
Cholesterol (mg/dl)	165.8±4.3
Triglycerides (mg/dl)	68.0±7.4

Values are means±standard error of means. ALT: alanine aminotransferase; AST: aspartate aminotransferase; BMI: body mass index; IAA: iso-alpha acids; LTA: lipoteichoic acid; PBMC: peripheral blood mononuclear cell

intake of the study drinks containing 15, 45, or 90 mg of IAA were stimulated with LTA for 6 h, the relative increase of IL-6 protein in cell supernatant compared to the fasted state (T0) was lower than after the intake of the placebo (Placebo: ~ + 1.7-fold; 15 mg IAA: ~ + 1.18-fold; 45 mg IAA: ~ + 1.38-fold; 90 mg IAA: ~ 0.956-fold) (see Fig. 1b). When cells isolated 2 h after the intake of the different IAA containing drinks were stimulated with LTA for 6 h, IL-6 protein levels were still lower when subjects had consumed the drink containing 90 mg of IAA. In contrast, IL-6 protein levels were slightly higher or similar in cell supernatant of cells isolated 2 h after the intake of 15 or 45 mg of IAA when compared to placebo. Also, 4 h and 6 h after the intake of the different IAA-enriched drinks, IL-6 protein levels in cell culture supernatant were either higher than after placebo consumption, or at the level of placebo (see Fig. 1b). No statistical differences were found for IL-6 protein concentrations in LTA-stimulated monocytes when comparing data between the interventions per time point (see Fig. 1b).

As the taste of IAA was perceived more tolerable by subjects at lower levels and effects were almost similar 1 h after IAA was consumed regardless of doses consumed, 15 mg of IAA was selected for all further in vivo studies as suitable IAA concentration and cells were isolated 1 h after the intake in the further human intervention study.

Effect of the oral intake of an IAA-rich hop extract on the LTA-induced cytokine release in healthy, normal weight men and women

To assess if similar effects as the ones found with respect to the LTA-induced release of IL-6 in monocytes are also found for other cytokines in men and women, we conducted a cross-over design study asking healthy women and men to consume the IAA-rich beverage (15 mg IAA derived through the IAA-rich hop extract) or a placebo in a randomized order with ingestions been separated by at least one week of washout. Characteristics of study participants are summarized in Table 2. Of the 17 subjects enrolled, 3 had to be excluded from the analysis as cells did not respond to the stimulation. Moreover, one subject discontinued the study after the first intervention. All of the participants were healthy and normal weight. Also, as cell yields were rather low an isolation of sufficient numbers of monocytes were not possible. Therefore, experiments were carried out with PBMCs.

Concentrations of IL-6 and IL-1 β protein were below the level of detection in cell supernatant of most subjects when cells were stimulated with vehicle. Therefore, these data are only shown for fasting samples (see Fig. 2b and c). In contrast, the challenge of PBMCs isolated from fasted subject before ingesting the different study drinks with LTA for 24 h resulted in a robust significant increase of IL-6 protein and IL-1 β protein levels in cell culture media (see Fig. 2b and c). In line with these findings, IL-6 and IL-1 β protein levels in cell supernatant of cells isolated 1 h after the ingestion of the placebo were similarly increased. In contrast, both IL-6 and IL-1 β protein levels in cell culture media of cells isolated 1 h after the intake of 15 mg IAA in the IAA-enriched study drink decreased significantly by ~31% and ~32% compared to controls (see Fig. 2b and c). In cell supernatant obtained from cells isolated 1 h after the intake of IAA and stimulated with LTA for 48 h, protein levels of IL-6 and IL-1 β were also significantly lower than in those obtained from cells isolated 1 h after the ingestion of placebo (placebo vs. IAA-rich drink: IL-6: ~ -37% and IL-1 β : ~ -32%, $p < 0.05$ for both differences) (see Fig. 2b and c).

Effect of the IAA-rich hop extract on the LTA-dependent induction of IL-6 and IL-1 β release, as well as TLR2 and MAPKinase signaling in primary human monocytes and J774A.1 cells

To determine if the effects found in vivo were related to direct effects of the IAA-rich hop extract on monocytes, we next challenged monocytes isolated from healthy donors with LTA in the presence of different, low doses of the IAA-rich hop extract (0–1.25 $\mu\text{g/ml}$ IAA). In line with the findings in vivo, the increase in IL-6 upon a 6 h long incubation with LTA was significantly attenuated when cells were pre-incubated for 1 h with 0.3125 $\mu\text{g/ml}$ and 0.625 $\mu\text{g/ml}$ IAA-rich hop extract before the challenge with LTA (see Fig. 3a). A similar trend was observed when cells challenged with LTA were concomitantly treated with 1.25 $\mu\text{g/ml}$ IAA ($p=0.0525$) (see Fig. 3a). The increase in IL-1 β protein concentration upon the challenge with LTA was significantly attenuated when cells were pre-treated for 1 h with 0.3125 $\mu\text{g/ml}$ of IAA-rich hop extract (see Fig. 3b). A similar trend was observed when cells were co-treated with 1.25 $\mu\text{g/ml}$ IAA-rich extract ($p=0.0784$) (see Fig. 3b).

As it has been suggested that some hop compounds (e.g., xanthohumol) dampen LTA-induced TLR2 signaling through interfering with the CD14-mediated transfer of the bacterial wall compound to TLR2 [11], we next determined if IAA also interfere with the LTA-dependent activation of TLR2 employing HEK-Blue™ TLR2 cell assay. Even when employing markedly higher concentrations than those used in vivo and shown to be effective in vitro in the present study, the preincubation of cells with IAA derived through the IAA-rich hop extract had no effect on the LTA-dependent activation of cells (see Fig. 4a).

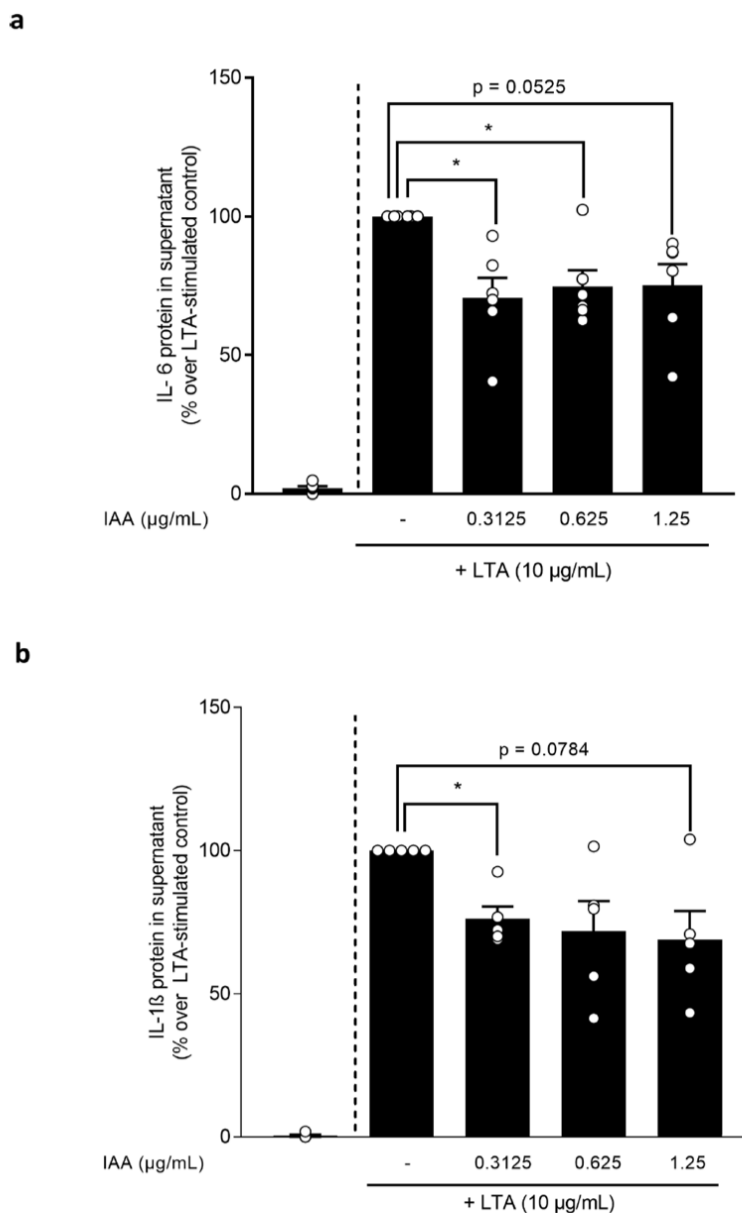
As results of others suggest that IAA may attenuate the activation of p38 and JNK [27], we next challenged J774.A1 cells with LTA for 6 h which resulted in a significant increase in the phosphorylation of both, p38 (see Fig. 4b) and JNK (see Fig. 4c). Results of pilot studies revealed that the IAA-rich hop extract attenuated the increase in IL-6 protein in cell culture supernatant derived from J774.A1 cells upon a stimulation with LTA in an almost dose-dependent way (see Supplemental Fig. 2); however, doses of the IAA-rich extract needed were markedly higher (0–25 $\mu\text{g/ml}$). Based on these experiments, 6.25 $\mu\text{g/ml}$ IAA derived through the hop extract was selected for all further experiments in J774.A1 cells (see Supplemental Fig. 2). The concomitant treatment of cells with 6.25 $\mu\text{g/ml}$ IAA and 5 ng/ml anisomycin, being a JNK and p38 agonist [30], when being challenged with LTA, attenuated the protective effects of IAA significantly (see Fig. 4d).

Discussion

The oral intake of low doses of IAA derived through an IAA-rich hop extract attenuated the LTA-dependent stimulation of PBMCs and monocytes ex vivo

Despite the availability of a large number of antibiotics, Gram-positive bacteria still pose a significant risk for bacterial infections in humans even in highly developed countries, causing a wide range of diseases such as skin infections, pneumonia, endocarditis, urinary tract infections, and life-threatening bloodstream infections [3, 31]. Indeed, in recent years the antibiotic resistance is becoming an increasing concern [32], often limiting treatment options and subsequently leading to complications and even death. Results of in vitro studies indicated that IAA derived from hop may dampen LPS-dependent activation of immune cells [13] but may also have antibacterial effects. It has been shown in vitro that IAA has ionophore properties disrupting cell membranes thereby leading, especially in Gram-positive bacteria, to cell lysis [33]. Epidemiological evidence supporting a protective effect of IAA consumption against Gram-positive infection, including infections caused by *Staphylococcus aureus*, *Streptococcus pneumoniae*, or *Enterococcus spp.*, is to our knowledge still lacking. In the present study, we determined the impact of an oral intake of 'nutritionally' relevant doses of IAA on the LTA-induced release of proinflammatory interleukins (e.g., IL-6 and IL-1 β) in primary monocytes and PBMCs isolated from young healthy adults. Concentrations of IAA used in present studies were based on those found in 250–1000 ml of commercially available Porter or Lager beer [34]. In the brewing process, alpha acids often also referred to as humulones, are isomerized to iso-alpha acids or isohumulones tasting rather bitter. In the present study, we used a standardized solution of iso-alpha acids (30% w/w) produced from CO₂ hop extract which when used in beer brewing is added to the beer after fermentation and before final filtration. While using lower doses than in most other studies in model organisms and cell cultures [35], we found that the LTA-dependent induction of the release of IL-6 protein from monocytes isolated from healthy women was markedly attenuated 1–2 h after the intake of the different IAA-rich drinks. Interestingly, results of our pilot data suggest that 1 h after the intake of all three drinks containing IAA effects were rather similar with respect to suppressing the release of IL-6 protein. This effect was lost when monocytes isolated 2 h after the intake of drinks containing 15 and 45 mg of IAA were stimulated with LTA while being persisted after the intake of the drink containing 90 mg of IAA. It has been shown before that IAA levels in blood increase as early as

Fig. 3 Effect of different concentrations of an iso-alpha acids rich hop extract on the LTA-dependent induction of IL-6 and IL-18 protein concentration in monocytes. Protein concentration of (a) IL-6 and (b) IL-18 in cell culture supernatant of monocytes isolated from healthy donors stimulated with 10 µg/ml LTA and different concentrations of IAA (0.3125–1.25 µg/ml) for 6 h. Data are presented as means ± SEM, $n = 5-6$. * $p < 0.05$. IAA: iso-alpha acids, IL: interleukin, LTA: lipoteichoic acid



30 min after the intake of beer but decline within 6 h back to baseline suggesting that IAA is rapidly taken up and metabolized [36, 37]. While not having been fully clarified yet, it has been suggested that iso-alpha acids are metabolized

through phase 1 (cytochrome P450) and 2 enzymes [38]. Our results are in line with results of previous studies of us and others showing in in vitro experiments and experiments in animals a more prolonged suppression of the release of

proinflammatory cytokines when employing higher doses of IAA [13, 25, 39]. In contrast to other studies, in the present study, we employed drinks to mimic the “nutrition” situation with dissolved IAA rather than capsulated IAA which would have been closer to a “pharmacological” setting. Still our approach was accompanied with the problem that IAA, which tastes rather bitter when consumed at higher concentrations, was fully tastable. Therefore, 15 mg were selected as a more suitable concentration of IAA to test effects in wider setting. In line with the findings of the pilot study, the oral intake of 15 mg of IAA derived through the IAA-rich hop extract suppressed the LTA-induced release of IL-6 and IL-1 β from isolated PBMCs. Taken together, these data suggest that the intake of low doses of IAA may decrease the immune response of blood immune cells to LTA in humans for 1–2 h after the intake. As in the present study cells were exposed to the bacterial toxin *ex vivo*, further studies are needed to assess if similar effect are also found when humans suffer from an infection with Gram-positive bacteria. Also, it remains to be determined, if effects found are persistent when IAA are consumed repetitively.

The effects of the IAA-rich extract on the LTA-dependent release of IL-6 are related to a suppression of an activation of JNK

Results of *in vitro* studies and animal models suggest that IAA may have direct effects on the inflammatory response of cells. Indeed, it has been shown before that in cell lines, like J774A.1 and L929sA, the pre-incubation with IAA before a challenge with bacterial endotoxin attenuates the release of IL-6 but also other proinflammatory mediators like RANTES and TNF α [13, 39]. These data suggest that the immunosuppressive effects of IAA could attenuate an excessive and uncontrolled immune response during bacterial infections. Further studies are needed to determine if the immune suppressive effects found in the present study and *in vitro* and *in vivo* studies of others translate into e.g., a shortening of the time being infected or the severity of an infection caused by bacteria in humans. Interestingly in these studies, concentrations of IAA used were mostly markedly higher than in the present study needed in primary cells obtained from healthy humans (+ 20–40-fold compared to the present study, for overview see [13]). Somewhat in line with the findings of others, in the present study, experiments carried out in J774A.1 cells also required markedly higher doses of IAA when compared to those showing effects in primary human monocytes. Indeed, it has been suggested before that while affecting similar signaling pathways, (murine) cells line might require other (higher) doses of compounds when compared to primary cells [40–42]. Also, it has been shown, that this might be related to the

species the cell lines were obtained from [43]. The protective effects of IAA with respect to inflammation have been related to an inhibition of the activation of NF κ B but also agonistic effect on PPAR α and γ (for overview see [35]). Moreover, it has been shown that MAPK and especially p38 (as well as JNK) are critical in the induction of IL-6 upon a challenge of cells with Gram-positive bacterial toxins such as LTA [44–46]. In the present study, the recognition of the Gram-positive bacterial toxin LTA by TLR2 was not altered as determined using stably TLR2 transfected HEK cells. The challenge of J774A.1 cells challenged with LTA for 6 h showed a massive activation of both p38 and JNK phosphorylation. Also, when treating cells with anisomycin, a p38 and JNK activator [30], the protective effects of IAA were abolished. These results somewhat contrast those of others showing no effect of rho iso-alpha acids on p38 and JNK phosphorylation in LPS-stimulated RAW 264.7 cells [47]. However, it could be that differences are related to the challenge e.g., the use of LTA in the present study and LPS in the study of others as well as the use of IAA vs. rho iso-alpha acids. In summary, our data suggest that IAA even at low doses can attenuated the LTA-dependent induction of IL-6 and IL-1 β protein release in primary human monocytes. Also, the protective effects of IAA seem to be related to altering JNK/p38-dependent signaling. Further studies are needed to determine if IAA has direct effects of JNK and p38 or if indirect effects are involved.

Limitations

Our study is not without limitations that need to be considered when interpreting the data. Specifically, while mostly employing primary human PBMCs and monocytes, all cell stimulation experiments were carried out *ex vivo*. Therefore, it remains to be determined if IAA, be they consumed as isolates or in an IAA-rich hop extract (30% w/w) as it was done in the present study, also affect the course of Gram-positive infections *in vivo* e.g., the length and the severity. Moreover, in the present study only acute effects resulting from a one-time intake of the IAA-rich extract were assessed. It remains to be determined if suppressing effects found with respect to alterations of bacterial toxin-dependent release of proinflammatory cytokines like IL-6 and IL-1 β are also found when IAA are regularly consumed. Moreover, as no other bitter component was used as a control in the placebo drink, and the bitter taste of the IAA-rich extract is difficult to mask, it cannot to be ruled out that subjects were able to distinguish not only the placebo from the IAA-enriched drinks but also the different IAA-containing drinks. Also, in the present study, we only assessed effects in healthy, normal weight humans. It remains to be determined if effects alike are also found in overweight and/ or older individuals.

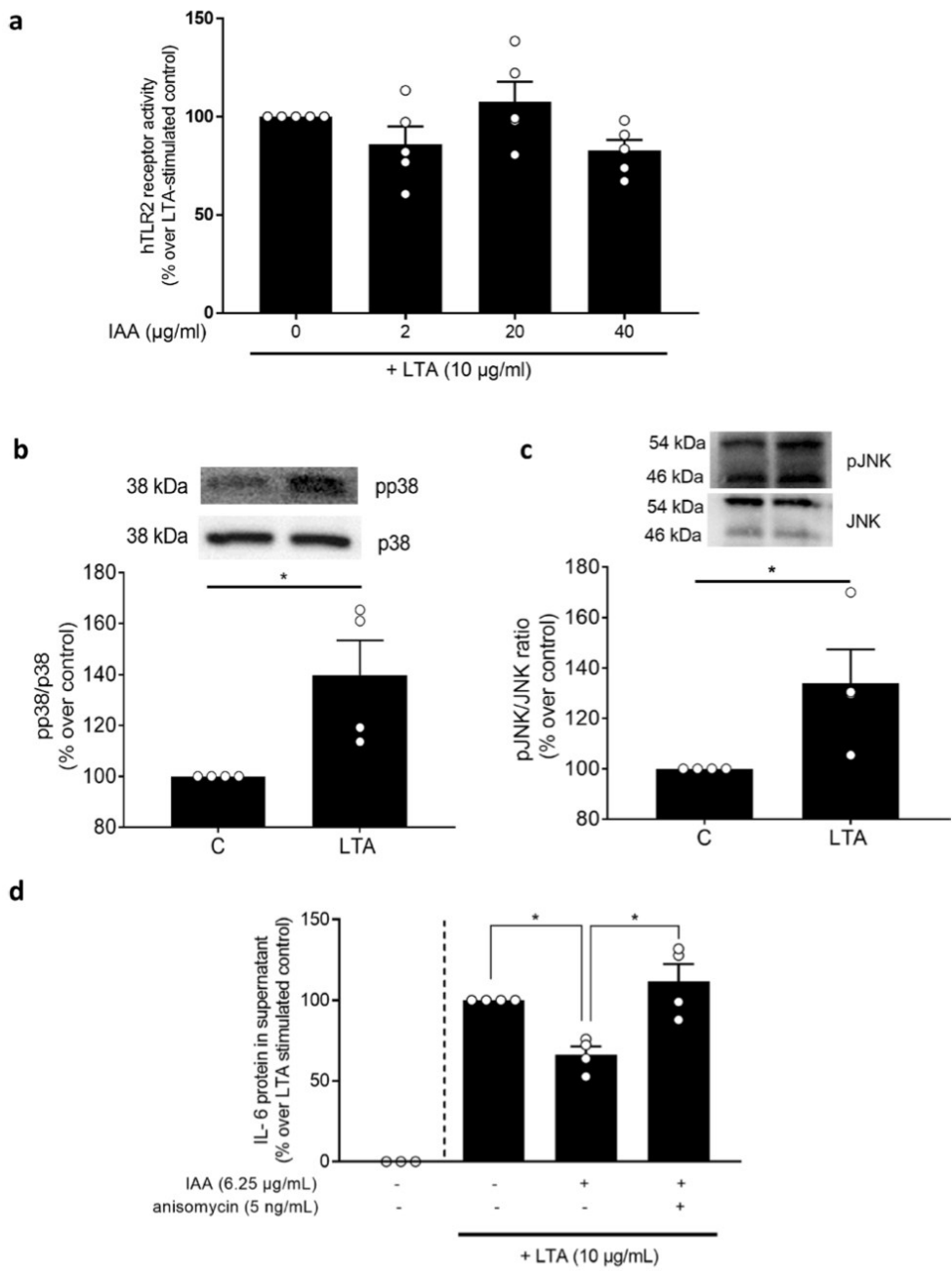


Fig. 4 Effect of an iso-alpha acids rich hop extract on the LTA-dependent induction of TLR2 and MAPKinase signaling in J774A.1 cells. **a** hTLR2 receptor activity from HEK293 cells co-incubated with 10 µg/ml LTA and increasing concentrations of IAA (0–40 µg/ml) for 12 h, **b** phospho-p38/p38 protein in J774A.1 cell stimulated with 10 µg/ml LTA for 6 h, **c** phospho-JNK/JNK protein in J774A.1 cells stimulated with 10 µg/ml LTA for 6 h and **d** IL-6 protein concentration in cell culture supernatant of J774A.1 cells stimulated with 10 µg/ml LTA and 0–6.25 µg/ml IAA rich hop extract and 0 or 5 ng/ml anisomycin for 6 h. Data are presented as means ± SEM, $n=4-5$. * $p<0.05$. IAA: iso-alpha acids, IL: interleukin, JNK: c Jun N terminal kinase, LTA: lipoteichoic acid, hTLR2: human Toll-like receptor 2

Moreover, in the present study, we used an IAA-rich hop extract which besides IAA contains other hop compounds that may have affected the outcome. However, at the time of study, no pure IAA suitable for the use in human nutrition was available.

Conclusion

Taken together, results of the present study suggest that an oral intake of IAA in low “nutritionally” relevant doses through an IAA-rich hop extract diminishes the LTA-dependent release of IL-6 and to a lesser extend also of IL-1 β from monocytes in humans ex vivo. Results of our study also suggest that the immune suppressing effects with respect to the release of IL-6 persist for ~1–2 h. Our results also suggest that these effects of IAA may be related to an inhibition of JNK and p38; however, further studies are needed to determine mechanisms underlying the interaction of IAA with stress-activated protein kinases. Also, it remains to be determined if an intake of IAA affects Gram-positive bacterial infections in vivo and if this interaction improves disease outcome in humans.

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Author contributions The authors responsibilities were as follows Katja Csarman: Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. Finn Jung, Anja Baumann, Benedikt Simbrunner, Kerstin Schweiger, Katharina Burger, Raphaela Staltner: Formal analysis, Investigation, Visualization, Writing – review & editing. Claus Hellerbrand: Formal analysis, Investigation, Visualization, Funding acquisition, Writing – review & editing. Ina Bergheim: Conceptualization, Supervision, Funding acquisition, Writing – original draft, Writing – review & editing.

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Data availability The original contributions presented in the study are

included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethical approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of University of Vienna, Vienna, Austria (reference numbers: 00367 and 00848) and were registered at clinical trials (NCT04847193, 16.03.2021; NCT06286644, 17.01.2024).

Consent to participate Informed consent was obtained from all individual volunteers included in the study.

Consent to publish Informed consent was obtained from all individual volunteers included in the study.

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2.4. Publication 4: Oral intake of low doses of an iso-alpha acid-rich hop extract dampens the LPS-mediated activation of blood immune cells: Randomized controlled trials in healthy individuals

Katja Csarmann, Finn Jung, Anja Baumann, Benedikt Simbrunner, Kerstin Schweiger, Katharina Burger, Raphaela Staltner, Claus Hellerbrand, Ina Bergheim.

Prepared for publication

Highlights:

- An oral dose of 15 mg IAA reduced LPS-induced IL-6 and IL-1 β release in monocytes one hour after the ingestion.
- PBMCs from IAA-treated participants showed significantly lower IL-6 and IL-1 β secretion after LPS stimulation compared to placebo.
- Effects of IAA were related to a suppression of LPS-dependent activation of TLR4/JNK signalling.

Contribution to the manuscript:

- Planning parts of the study and preparing the ethics application
- Conducting parts of the human intervention study
- Conducting the *ex vivo* stimulation experiments
- Performing laboratory chemical analyses of human samples
- Analysing the data and performing statistical evaluations
- Preparing tables and figures and drafting the manuscript

Total contribution: 30%

1 **Oral intake of low doses of an iso-alpha acid-rich hop extract dampens the LPS-**
2 **mediated activation of blood immune cells: Randomized controlled trials in healthy**
3 **individuals**

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25 Ina Bergheim is an Editorial Board Member for *The Journal of Nutrition*. She played no role in
26 the Journal's evaluation of this manuscript.

27 List of abbreviations

28 AP-1, activating protein-1; ANOVA, Analysis of Variance; ALT, Alanine aminotransferase;
29 AST, Aspartate aminotransferase; BMI, Body Mass Index; CD14, Cluster of Differentiation 14;
30 DMEM, Dulbecco's Modified Eagle Medium; ELISA, Enzyme-Linked Immunosorbent Assay;
31 HEK, Human Embryonic Kidney (cells); HRP, Horseradish Peroxidase; IAA, Iso-alpha acids;
32 IL, Interleukin; IKK β , Inhibitory KB kinase β , JNK, c-Jun N-terminal kinase; LPS,
33 lipopolysaccharide; MAPK, Mitogen-Activated Protein Kinase; NF κ B, Nuclear Factor kappa-
34 light-chain-enhancer of activated B cells; PBMC, Peripheral Blood Mononuclear Cells; RIPA
35 buffer, Radio-Immunoprecipitation assay buffer; RPMI, Roswell Park Memorial Institute
36 (medium); SEM, Standard Error of the Mean; TLR, Toll-like receptor; TNF, tumor necrosis
37 factor

38 Abstract

39 **Background:** Iso-alpha acids (IAA) found in hop (*Humulus lupulus L.*) have shown anti-
40 inflammatory effects *in vitro* and in animal models. **Objective:** To assess if 'nutritional' doses
41 of IAA derived through an IAA-rich hop extract affect inflammatory process in humans, two
42 randomized, single-blinded, placebo-controlled human intervention studies were performed.
43 **Methods:** To determine dose- and time-dependent effects monocytes were isolated from
44 blood of 5 healthy female volunteers after an oral intake of 0-90 mg IAA derived through an
45 IAA-rich hop extract and cells were challenged with LPS *ex vivo* to determine IL-1 β and IL-6
46 protein release. In a second study, peripheral blood mononuclear cells (PBMCs) were isolated
47 from 10 healthy men and women after the intake of 15 mg IAA derived through an IAA-rich hop
48 extract and the LPS-dependent release of IL-1 β , IL-6 and TNF- α was measured. Moreover,
49 J774A.1 were challenged with LPS in the presence of IAA and the JNK activator anisomycin.
50 **Results:** The results of the pilot study in healthy women showed that the LPS-induced IL-1 β
51 and IL-6 release from monocytes was reduced when the cells were isolated one hour after the
52 ingestion of 15 mg IAA from an IAA-rich hop extract. In a second study, the intake of 15 mg
53 IAA significantly suppressed the release of IL-1 β and IL-6 from PBMCs isolated from healthy
54 men and women. Effects of IAA on LPS-dependent inflammation were related to a suppression
55 of LPS-dependent activation of TLR4/JNK signaling. **Conclusion:** These findings provide the
56 first evidence that the low-dose IAA intake modulates inflammatory responses of immune cells
57 in humans.

58

59 **Keywords:** Iso-alpha acids; lipopolysaccharide; Toll-like receptor 4; cytokines; *Humulus*
60 *lupulus L.*

61 Introduction

62 Bacterial infections are a major global health concern. In 2019, infections caused by 33
63 bacterial pathogens were the second leading cause of deaths worldwide [1]. The majority of
64 these infections were caused by the Gram-positive bacterium *Staphylococcus aureus* and by
65 the Gram-negative bacterium *Escherichia coli* [2]. Indeed, resistance to antibiotics used for the
66 treatment of Gram-negative bacterial infections has increased dramatically during the last 30
67 years, resulting in an increase of associated deaths from 619 000 in 1990 to 1 million in 2021
68 [3]. It is estimated that by 2050, 8 million deaths will be associated with antibacterial multidrug
69 resistance globally [3]. Components of the outer cell wall of Gram-negative bacteria like
70 lipopolysaccharides (LPS) play a key role in triggering the development of inflammatory
71 responses and immune disturbances, which can lead to severe infections [4, 5]. The activation
72 of Toll-like receptor 4 (TLR4), which recognises LPS, has been linked not only to bacterial
73 infections but also to the progression of chronic conditions, including metabolic dysfunction-
74 associated steatotic liver disease, alcohol-related liver diseases and type 2 diabetes [6-8].
75 TLR4 has been shown to bind bacterial LPS through a complex interaction with cluster of
76 differentiation 14 (CD14) [9]. The LPS-induced activation of the TLR4 signaling cascades leads
77 to a significant cytokine upregulation, including interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and
78 tumor necrosis factor α (TNF- α) [10].

79 Studies suggest that secondary plant compounds found in hops, such as α -acids, β -acids, and
80 xanthohumol, may exhibit anti-inflammatory properties in various disease contexts [11, 12]. In
81 the brewing process, hops are not only valued for their flavour but also for their antimicrobial
82 properties [13]. Animal studies indicate that ingesting iso-alpha acids (IAA), which are derived
83 from alpha-acids through thermal isomerisation [24], can mitigate inflammatory responses,
84 including the production of proinflammatory cytokines and oxidative stress in liver and brain
85 tissue, with these effects observed in models of alcohol- and diet-induced liver damage as well
86 as Alzheimer's disease [14-18]. *In vitro* studies also indicate that IAA may affect nuclear factor
87 kappa-light-chain-enhancer of activated B cells (NF κ B) and activating protein-1 (AP-1)

88 activation by modulating the c-Jun N-terminal kinase (JNK) and inhibitory KB kinase β (IKK β)
89 pathways [17, 19]. However, it is unclear whether IAA, which is typically consumed in low
90 doses when physical doses of alcohol-free or alcohol-containing beers are consumed, has
91 beneficial effects in humans such as attenuating the inflammatory responses triggered by
92 Gram-negative bacteria.

93 Starting from this background, the primary aim of the present study was to first determine if a
94 'physiological' oral dose of IAA derived through an IAA-rich hop extract, affects the immune
95 response in blood monocytes in young healthy women, and the duration of these effects. In a
96 second randomized, placebo-controlled crossover trial in a mixed-gender cohort, the impact of
97 a single dose of IAA-rich hop extract (15 mg IAA) or placebo was evaluated in young healthy
98 individuals, and related molecular mechanisms were examined.

99 **Materials and Methods**

100 **Human intervention studies**

101 All intervention studies were carried out in accordance with the ethical standards laid down in
102 the Declaration of Helsinki of 1975 as revised in 1983, using a randomized placebo-controlled,
103 crossover design. The studies were approved by the Ethics Committee of the University of
104 Vienna, Vienna, Austria (reference numbers: 00367, 00848) and were registered at
105 ClinicalTrials.gov (NCT04847193, 16.03.2021; NCT06286644, 17.01.2024). Written informed
106 consent was obtained from all participants before they were enrolled in the study. The following
107 exclusion criteria were defined for both studies: (1) following a special diet or (2) food
108 malabsorption or (3) a history of gastrointestinal diseases. The following inclusion criteria were
109 also defined: normal weight ($\text{BMI} > 18.5 \text{ kg/m}^2$ or $< 24.9 \text{ kg/m}^2$), non-smoking status, and no
110 viral or bacterial infection 3 weeks prior to the study. The use of anti-inflammatory medication
111 was also defined as an exclusion criterion for the studies. In both studies, the participants were
112 asked to refrain from consuming hop products for two weeks prior to the studies and during
113 the intervention.

114 Time- and dose-response pilot study: To determine the optimal time for cell isolation and the
115 appropriate IAA doses, a single-blinded, randomized placebo-controlled pilot study was
116 conducted with normal weight, healthy female volunteers. For the preparation of the study
117 drink, 10 ml of water, freshly prepared just before the intake, thickener (Nestlé S.A., Vevey,
118 Switzerland), sugar-free lemon flavour (SodaStream GmbH, Frankfurt am Main, Germany),
119 and 0, 15, 45 or 90 mg IAA derived from an IAA-rich hop extract (Isohop®, generously provided
120 by Barth-Haas Group GmbH & Co. KG, Nuremberg, Germany) were mixed together. After an
121 overnight fast and blood sampling, participants were asked to consume the study drink within
122 15 minutes along with a standardised breakfast (2 medium-sized pretzels with 30 g butter).
123 Blood samples were taken 1, 2, 3, 4 and 6 h after consumption of the study drink. Between the
124 4 and 6 h time points, another standardized meal was provided. After the first study day and a
125 1-week washout phase, during which participants were instructed to refrain from all hop-

126 containing foods and beverages, the intervention was repeated in a cross-over design until all
127 four study days were completed. The study design and the procedure on the study day are
128 summarized in Figure 1a. Clinical parameters were determined in the serum of the participants
129 and monocytes were isolated from whole blood as detailed below.

130 Assessment of the acute effects of IAA: In a follow-up study, based on the results from the
131 time- and dose-response study, the effect of 15 mg of IAA derived from an IAA-rich hop extract
132 was evaluated compared to a placebo. Male and female participants were randomly assigned
133 in a single-blinded manner to receive a study drink, as described earlier, containing either 0 or
134 15 mg of IAA from the hop extract. The study drink was consumed within 15 minutes along
135 with a standardised breakfast (2 medium-sized pretzels with 30 g of butter). Participants
136 underwent an overnight fast, with blood samples collected both before the consumption of the
137 study drink and one hour after ingestion. Following the first study day and a 1-week washout
138 phase, where participants were instructed to avoid all hop-containing foods and beverages,
139 the intervention was repeated in a cross-over design. The study design and the procedures on
140 the study day are summarized in Figure 2a. Clinical parameters were analysed in the serum
141 of the participants and peripheral blood mononuclear cells (PBMCs) were isolated from whole
142 blood as described below.

143 For basic characteristics of the study subjects of the two studies see Table 1 and Table 2.

144 **Monocyte isolation**

145 Monocytes were isolated from whole blood samples collected from participants of the time-
146 and dose-response pilot study. The isolation of cells was performed using density gradient
147 centrifugation with the pluriSelect system, following the manufacturer's protocol (pluriSelect
148 Life Science UG & Co. KG, Leipzig, Germany). Briefly, blood was layered onto monocyte
149 density gradient media (1.068 g/ml) in separation tubes (pluriSelect Life Science UG & Co.
150 KG, Leipzig, Germany) and centrifuged at room temperature for 15 minutes. The cells were
151 then collected and prepared for further experiments as described below.

152 **PBMCs isolation**

153 PBMCs were isolated from whole blood samples obtained from participants of the study, in
 154 which the acute effects of IAA were assessed. Cells were isolated by density gradient
 155 centrifugation. In brief, blood was layered onto Pancoll solution (1.077 g/ml) (Pan-Biotech
 156 GmbH, Aidenbach, Germany) and centrifugated for 30 minutes at room temperature. Cells
 157 were collected and used for further experiments as described below.

158 **Monocytes and PBMCs stimulation obtained from the pilot study and acute effects** 159 **study after an oral ingestion of an IAA-rich hop extract**

160 Isolated monocytes and PBMCs were cultivated in RPMI-1640 medium (Sigma-Aldrich
 161 Chemie GmbH, Steinheim, Germany) with 10% fetal bovine serum (Pan-Biotech GmbH,
 162 Aidenbach, Germany) and 100 µg/ml streptomycin and 100 U/ml penicillin (Pan-Biotech
 163 GmbH, Aidenbach, Germany) at 37 °C in a humidified 5% CO₂ atmosphere for 1 h.
 164 Subsequently, cells were either challenged with 0 or 100 ng/ml LPS for 24 h for the Western
 165 Blot analysis or for 6 h for all other experiments. Cell culture supernatant was collected at the
 166 end of the experiment and stored at -80 °C until further use.

167 **Cell culture experiments with hTLR4 transfected HEK cells**

168 To assess the effects of IAA on TLR4 ligand concentration, a commercially available reporter
 169 gene assay with HEK-Blue™ TLR4 cells was used (InvivoGen, Toulouse, France). Following
 170 the instructions of the manufacturer, cells were grown in a humidified, 5% CO₂ atmosphere
 171 and were grown up to 80% confluency using DMEM media (Pan-Biotech GmbH, Aidenbach,
 172 Germany). Cells were challenged with 5 ng/ml LPS for 16 h in the presence of 0–10 µg/ml IAA
 173 dissolved in HEK Blue™ Detection medium (InvivoGen, Toulouse, France). Colour changes of
 174 the medium being indicative of ligand concentration and binding were determined at 655 nm.

175 **Cell culture experiments with J774A.1 cells**

176 J774A.1 cells (DMSZ, Braunschweig, Germany), which exhibit a morphology similar to that of
 177 macrophages/monocytes, were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Pan-

178 Biotech GmbH, Aidenbach, Germany) supplemented with 10% fetal bovine serum (Pan-
179 Biotech GmbH, Aidenbach, Germany), 100 µg/ml streptomycin, and 100 U/ml penicillin (Pan-
180 Biotech GmbH, Aidenbach, Germany) at 37°C in a humidified 5% CO₂ atmosphere. Once the
181 cells reached 80% confluency, they were preincubated for 1 h with or without 1 ng/ml
182 anisomycin, followed by another 1 h preincubation with or without 2.5 µg/ml IAA. After this, the
183 cells were treated with 50 ng/ml LPS for 6 h. The cells were lysed and the supernatant was
184 collected at the end of the experiment and stored at -80°C for further use.

185 **Enzyme-Linked Immunosorbent Assays (ELISA's)**

186 IL-1β, IL-6 and TNF-α protein concentrations were analysed in cell culture supernatant of
187 monocytes and PBMCs or J774A.1 cells using commercially available DuoSet® ELISA
188 Development Systems (R&D®, Minneapolis, USA) kits.

189 **Western Blot Analysis**

190 To determine JNK activation, J774A.1 cells were homogenized in RIPA buffer (20 mmol/l 3 [N-
191 morpholino] propanesulfonic acid, 150 mmol/l NaCl, 1 mmol/L ethylenediaminetetraacetic acid,
192 1% Nonidet P-40, and 0.1% sodium dodecyl sulfate) containing protease and phosphatase
193 inhibitor cocktails (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) to obtain protein
194 lysates. To determine CD14 protein, cell culture supernatant from stimulated PBMCs of the
195 acute effects study were used for the Western Blot analysis. Protein lysates (5 µg/lane) and
196 cell culture supernatant of PBMCs from the acute study was separated on 10% SDS-
197 polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Bio-Rad
198 Laboratories, Hercules, CA, USA). Membranes were further incubated with specific primary
199 antibodies (phosphorylated c-Jun N-terminal kinase (JNK) and total JNK and CD14 (Cell
200 Signaling Technology, MA, USA)) and the respective secondary antibody (anti-rabbit IgG,
201 HRP-linked; Cell Signaling Technology, MA, USA). To detect the protein bands, Clarity
202 Western Enhanced Chemiluminescence Substrate (Bio-Rad Laboratories, Hercules, CA,
203 USA) and ChemiDoc XRS System (Bio-Rad Laboratories, Hercules, CA, USA) were used.

204 Densitometric analyses of detected bands were performed with ImageLab (Bio-Rad
205 Laboratories, Hercules, CA, USA).

206 **Statistical Analyses**

207 Data are presented as means $\pm$ standard error of the means (SEMs). Grubb's test was
208 performed before statistical analysis to identify outliers. Homogeneity of variances was tested
209 and data were log-transformed if data were not normal distributed or in case of inhomogeneity
210 of variances before performing further statistical tests. A t-test was used to determine
211 statistically significant differences between two groups and one-way ANOVA was used for all
212 other comparisons (GraphPad Prism Software, CA, USA). A p-value < 0.05 was considered to
213 be significant.

214 **Results**

215 **Time- and dose-dependent effects of an acute oral intake of an IAA-rich hop extract on**
 216 **the LPS-induced IL-1 β and IL-6 release in monocytes: Pilot study**

217 To determine whether an oral intake of IAA derived through an IAA-rich hop extract has an
 218 effect on cytokine release in blood immune cells, we conducted a pilot study in 5 healthy,
 219 normal weight women. Participant characteristics are summarized in Table 1. The study design
 220 and procedure on the study day are summarized in Figure 1a. As expected, challenging
 221 monocytes with LPS isolated before and 1 h after the intake of the placebo resulted in a ~1.5-
 222 fold increase in both IL-1 β and IL-6 protein levels (see Supplemental Figure 1a and 1b). In
 223 contrast, when cells were isolated 1 h after the intake of the study drinks containing 15 mg, 45
 224 mg or 90 mg of the IAA-rich hop extract, the relative LPS-induced IL-1 β and IL-6 protein release
 225 was lower compared to fasted state (T0) than after the intake of the placebo (see Figure 1b
 226 and 1c). This effect persisted for up to 2 h for IL-1 β when subjects had consumed the 45 mg
 227 IAA but was not found after the intake of the drinks enriched with 15 mg and 90 mg of IAA,
 228 respectively. Three, 4 and 6 h after the intake of the different IAA-enriched drinks, IL-1 β protein
 229 levels in cell culture supernatant were either at the level of placebo, or higher than after placebo
 230 consumption (see Figure 1b). In contrast, IL-6 release was lower or at the level of placebo after
 231 2 h after the intake of the different IAA-enriched drinks (see Figure 1c) but this was not found
 232 3 and 4 h after the intake of the different IAA-enriched drinks. Six hours after the intake of the
 233 different IAA-enriched drinks, IL-6 levels were either at the level of placebo or lower than after
 234 placebo consumption (see Figure 1c); however, generally, data varied considerably. As
 235 subjects found the taste of IAA containing beverage more tolerable at lower doses and, as the
 236 effects were most pronounced 1 h after consuming 15 mg IAA, we selected the lowest IAA
 237 concentration and 1 h for all further studies.

238 **Effects of an acute oral intake of 15 mg IAA in an IAA-rich hop extract on the LPS-**
 239 **induced cytokine release in healthy, normal weight men and women**

240 To determine whether the observed effects on LPS-induced IL-1 β and IL-6 release in
241 monocytes were also evident for other cytokines (e.g., TNF- α) and in male subjects, we
242 conducted a randomized cross-over design study in healthy, normal weight men and women
243 (study design see Figure 2a). Participant characteristics are summarized in Table 2. Of the 14
244 subjects enrolled originally, 4 were excluded from the analysis due to an insufficient number
245 of cells for stimulation or a lack of response to the cell stimulation at one or more timepoints.
246 Moreover, as monocyte yield was too low to measure all three cytokines (IL-1 β , IL-6, TNF- α),
247 we conducted the study with PBMCs instead. As cytokine concentrations were below the level
248 of detection in cell supernatant of most subjects when cells were stimulated with vehicle, these
249 data are shown as not detectable (see Figure 2b, 2c and 2d). The challenge of PBMCs isolated
250 before the study drink ingestion (fasting) with LPS for 6 h resulted in a robust increase in IL-
251 1 β , IL-6 and TNF- α protein in cell supernatant compared to unstimulated cells. In line with the
252 findings of the pilot study, IL-1 β protein levels in cell supernatant 1 h after the ingestion of 15
253 mg of the IAA-rich hop extract was significantly lower compared to placebo by ~57% ($p < 0.05$,
254 see Figure 2b). Similar effects were also found in IL-6 protein levels in cell supernatant 1 h
255 after the ingestion of 15 mg of IAA-rich hop extract compared to placebo (~38% compared to
256 placebo, $p < 0.05$, see Figure 2c). In contrast, TNF- α protein levels were not altered (see Figure
257 2d).

258 ***In vitro* studies assessing the effects of IAA on the LPS-dependent activation of CD14,**
259 **TLR4 and JNK pathways**

260 As it has been suggested that some hop compounds, like xanthohumol, dampen LPS-induced
261 TLR4 signaling through interfering with the CD14-mediated transfer of the bacterial wall
262 compound to TLR4 [11], we next determined if IAA also affects the interaction of LPS and
263 CD14 or TLR4. Neither the intake of the different study drinks nor the challenge of cells with
264 LPS for 6 h altered CD14 protein levels in cell supernatant (see Figure 3a and 3b,
265 Supplemental Figure 2). In contrast, the LPS-induced activation of TLR4-transfected HEK293
266 cells was nearly dose-dependently inhibited when the cells were exposed to the IAA-rich hop

267 extract at concentrations between 0 and 10 $\mu\text{g/ml}$ (see Figure 3c). As results of others suggest
268 that IAA may attenuate the activation of JNK [19], having been shown to be activated through
269 TLR4-dependent mechanisms [20], we next challenged J774A.1 cells with LPS in the presence
270 and absence of low doses of IAA. Based on the studies in TLR4-transfected HEK293 cells and
271 an initial dose-response analysis which suggested that the IAA-rich hop extract at
272 concentrations of 2.5 $\mu\text{g/ml}$ IAA reduced the TLR4 activity by $\sim 30\%$ (see Figure 3c) and the
273 release of IL-1 β from J774A.1 cells by $\sim 69\%$ (see Supplemental Figure 3), 2.5 $\mu\text{g/ml}$ IAA was
274 selected as suitable concentration for the experiments in J774A.1 cells. The increase in JNK
275 phosphorylation induced by LPS was almost completely attenuated in cells concomitantly
276 treated with IAA (see Figure 4a and 4b, Supplemental Figure 4). Moreover, the decrease of
277 TNF- α and IL-6 protein levels in cells treated with LPS in the presence of IAA was significantly
278 attenuated when cells were concomitantly exposed to anisomycin being an agonist of JNK and
279 p38 [21] (see Figure 4c and 4d).

280 Discussion

281 Gram-negative bacteria continue to represent a considerable clinical challenge, even in high-
282 income countries, due to their capacity to cause a broad spectrum of infections, including
283 urinary tract infections, pneumonia, intra-abdominal infections and bacteraemia [22, 23]. The
284 rapid emergence of multidrug-resistant strains, particularly *E. coli*, *K. pneumoniae*, *P.*
285 *aeruginosa* and *A. baumannii*, severely constrains therapeutic options and is associated with
286 poor clinical outcomes, prolonged hospital stays and increased mortality [24-26]. Resistance
287 to last-line agents like carbapenems and polymyxins is linked to high morbidity and mortality
288 [26, 27], underscoring the urgent need for alternative therapeutic approaches. Here, we
289 assessed the effect of a 'nutritional dose' of IAA administered through the intake of an IAA-
290 rich hop extract on the release of the proinflammatory cytokines IL-1 β , IL-6 and TNF- α upon a
291 stimulation of primary monocytes and PBMCs isolated in young healthy women and men. The
292 concentrations of iso-alpha acids employed in our study were selected to reflect levels typically
293 present in 250-1000 ml of commercially available Porter or Lager beers [28] but also alcohol-
294 free beer. During brewing, hop-derived alpha acids (also referred to as humulones) undergo
295 isomerization to iso-alpha acids (isohumulones), which confer the characteristic bitter taste of
296 beer [29]. For our experiments, we used a commercially in brewing used iso-alpha acid rich
297 hop extract (30% w/w) obtained through standardized extraction method from hop. In beer
298 production, this preparation is usually added after fermentation and prior to final filtration to
299 modify the taste of beer. Using lower IAA doses than those commonly applied in studies with
300 model organisms or cell cultures [30], we observed that LPS-induced IL-1 β and IL-6 release
301 from monocytes isolated from healthy women 1 to 6 h after the intake of the IAA-enriched
302 drinks was markedly lower 1 h after intake of the IAA-rich drinks. Notably, one hour after
303 consumption, all three IAA-containing drinks produced a suppressive effect on IL-1 β and IL-6
304 secretion. This effect was no longer evident for IL-1 β two hours after the intake of drinks
305 containing 15 or 90 mg of IAA, whereas it persisted following the drink containing 45 mg of
306 IAA; however, data varied considerably. The reason for the slightly longer immune suppressive
307 effect found after the intake of 45 mg of IAA on IL-1 β release remains to be determined. Studies

308 in humans have shown before, that upon ingestion of beer (600 - 800 ml, 40 mg/l IAA)
309 containing concentrations similar to those used in the present study, IAA concentrations
310 increase rapidly peaking at ~30-60 minutes followed by a decline until 6 h after the intake [31].
311 The measured blood concentrations of IAA in these volunteers were about 0.1 mg/l 30 minutes
312 after the beer consumption. Although the metabolic pathways of IAA in humans are not yet
313 fully elucidated, it has been proposed that they undergo biotransformation via phase I
314 (cytochrome P450) and phase II enzymes [32]. Our findings are consistent with previous *in*
315 *vitro* and animal studies, including our own work, which demonstrated a more sustained
316 suppression of proinflammatory cytokine release at higher IAA doses [16, 17]. Unlike other
317 studies, in the present study an IAA-enriched drink was used to better reflect a nutritional
318 context, rather than using encapsulated preparations that would resemble a more
319 pharmacological setting. However, IAA are distinctly bitter at higher concentrations, therefore
320 limiting their use in an isolated form in drinks. To enable testing in a broader setting, 15 mg
321 was therefore selected as a more feasible concentration. Consistent with our pilot findings, 1
322 h after the oral intake of 15 mg IAA from the hop extract, LPS-induced release of IL-6 and IL-
323 1 β protein from isolated PBMCs was suppressed. Interestingly, while concentrations of TNF-
324 α were also slightly lower, these differences did not reach the level of significance. Studies
325 suggest that TNF- α expression and release is time-dependent and cell-specific [33-35]. It could
326 be that as in the present study only one time point was used to assess the effects of IAA on
327 cytokine release that the timepoint for TNF- α was missed. It could also be that IAA affected
328 signaling cascades are more critical in the regulation of IL-6 and IL-1 β than TNF- α . [36]. This
329 needs to be determined in further studies. Taken together, our data suggest that even low
330 doses of IAA can transiently reduce the responsiveness of human blood immune cells to LPS
331 for up to 1-2 h after intake. Since cells in this study were exposed to bacterial endotoxin *ex*
332 *vivo*, future research is needed to determine whether similar effects occur during infections
333 with Gram-negative bacteria. Moreover, it remains to be clarified whether these
334 immunomodulatory effects persist with repeated IAA consumption and how the response to a
335 challenge with lower LPS concentrations is.

336 **The immune-suppressive effects of the IAA-rich hop extract on the LPS-dependent**
337 **proinflammatory cytokine release is related to a suppression of JNK activation**

338 *In vitro* studies and animal studies employing high doses of IAA suggest that IAA possibly may
339 directly impact the inflammatory response in cells [16, 18, 37]. For instance, previous studies
340 have demonstrated that pre-incubating cell lines such as J774A.1 and L929sA with IAA can
341 reduce the release of proinflammatory cytokines when exposed to bacterial endotoxin [16, 37].
342 Our findings support the hypothesis that IAA interferes with the LPS and TLR4-dependent
343 activation of JNK. Indeed, somewhat in line with the finding for xanthohumol [11], the activation
344 of TLR4 by its ligand LPS was almost dose-dependently attenuated; however, contrasting the
345 findings for xanthohumol, the intake of IAA ingestion had no effect on CD14 protein levels in
346 cell supernatant obtained from LPS-stimulated PBMCs isolated 1 h after the intake of the study
347 drinks. The suppressive effects of IAA on the LPS-dependent activation of TLR4 was related
348 with a normalisation of JNK activation in J774A.1 cells which was attenuated when the
349 JNK(p38) agonist anisomycin was present. Indeed, in the presence of 1 ng/ml anisomycin the
350 IAA-related suppression of the LPS-induced release of TNF- α and IL-6 was almost completely
351 attenuated. These results are contrasting those of others reporting no effect of Rho-iso-alpha
352 acids on JNK phosphorylation in LPS-stimulated RAW 264.7 cells [38]. Differences between
353 the results of the present study and those of others could be related to differences in the IAA
354 used (here an IAA-rich hop extract vs a Rho-iso-alpha extract in the study of others). This
355 needs to be further studied in future studies. In summary, our data suggest that the
356 suppression of the TLR4/JNK signaling cascade is a key mechanism of the anti-inflammatory
357 response induced by IAA. Nevertheless, our findings do not preclude the possibility that iso-
358 alpha acids may also influence other signaling cascades, particularly when consumed in higher
359 doses or over a longer period of time. Rather, our results suggest that IAA obtained from an
360 IAA-rich hop extract may, when consumed in non-pharmacological concentrations, at least
361 temporarily attenuate the TLR4 signaling cascade in blood immune cells. Also, further studies
362 are needed to determine how IAA alters TLR4 activity.

363 In summary, the findings of this study suggest that the oral intake of low, 'nutritional' doses of
364 IAA in the form of an IAA-rich hop extract, reduces the release of IL-1 β from primary human
365 monocytes *ex vivo* in response to LPS, with a more modest effect on IL-6. The immune-
366 suppressing effects on IL-1 β appear to last for approximately 1-2 h. Our results also suggest
367 that these effects of IAA may be linked to a suppression of the TLR4 activation and the JNK
368 pathway. However, further research is needed to fully elucidate the mechanisms by which IAA
369 interacts with TLR4 and subsequently stress-activated protein kinases. Furthermore, it remains
370 to be determined whether IAA intake influences the progression of Gram-negative bacterial
371 infections *in vivo*, and whether such interactions alter disease outcomes in humans.

372 Our study has some limitations that should be considered when interpreting the results. Firstly,
373 while we primarily used human PBMCs and monocytes, all cell stimulation experiments were
374 conducted *ex vivo*. Consequently, it remains unclear whether IAA, when consumed as an
375 isolate or through IAA-rich hop extract (30% w/w) as in the present study, would also have
376 effects on patients with bacterial infections caused by Gram-negative bacteria. Additionally,
377 this study only examined the acute effects of a single dose of the IAA-rich extract. It is unclear
378 whether observed suppression of bacterial toxin-induced release of proinflammatory cytokines
379 such as IL-1 β , IL-6 and TNF- α would persist when IAA is consumed regularly. Moreover, as
380 no 100% pure IAA solution, for the use in humans is available, it can also not be ruled out that
381 other compounds found in the IAA-rich hop extract affected the outcome of the present study.
382 Furthermore, as we only assessed the effects of IAA in healthy, normal weight individuals, it is
383 unclear whether similar results would be observed in overweight or older populations.

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386 Ethics approval

387 This study was performed in line with the principles of the Declaration of Helsinki. Approval
388 was granted by the Ethics Committee of University of Vienna, Vienna, Austria (reference
389 numbers: 00367 and 00848) and were registered at clinical trials (NCT04847193;
390 NCT06286644).

391 Informed consent statement

392 Informed consent was obtained from all individual volunteers included in the study.

393 Author contributions

394 KC: Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review &
395 editing. FJ, AB, BS, KS, KB, RS: Formal analysis, Investigation, Visualization, Writing – review
396 & editing. CH: Formal analysis, Investigation, Visualization, Funding acquisition, Writing –
397 review & editing. IB: Conceptualization, Supervision, Funding acquisition, Writing – original
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402 Data availability statement

403 The original contributions presented in the study are included in the article/ Supplementary
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406 Conflict of interest

407 The authors declare that they have no conflict of interest. The funders and participants had
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- 535

536 **Table 1. Characteristics of healthy female subjects enrolled in the dose- and time-**
 537 **response study.**

Parameter	Baseline
Sex (m/f)	0/5
Age (years)	26.4 ± 1.7
Body weight (kg)	56.1 ± 0.8
Height (m)	1.7 ± 0.0
BMI (kg/m ²)	20.4 ± 0.7
Fasting glucose (mg/dl)	86.0 ± 3.4
AST (U/L)	22.6 ± 3.4
ALT (U/L)	17.0 ± 3.3
Cholesterol (mg/dl)	160.4 ± 9.8
Triglycerides (mg/dl)	65.6 ± 4.1

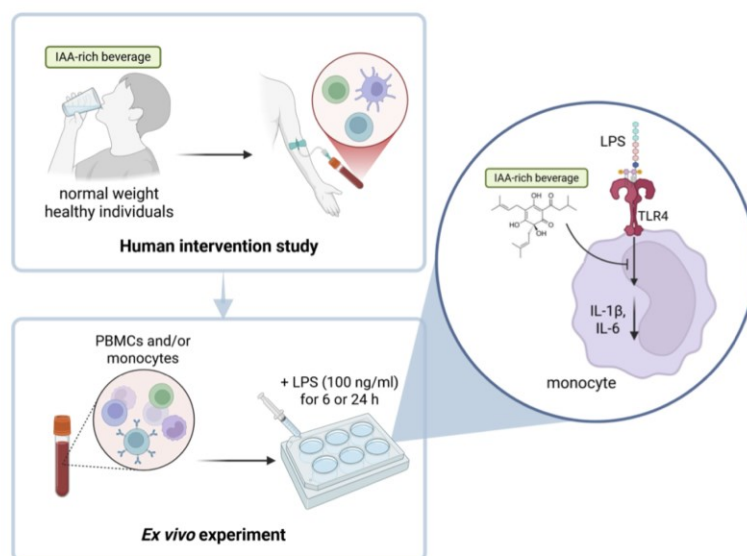
538 Values are means ± standard error of means. BMI: body mass index; ALT, alanine
 539 aminotransferase; AST, aspartate aminotransferase.

540 **Table 2. Characteristics of healthy subjects enrolled in the study to assess the acute**
 541 **effect of IAA on LPS-dependent immune response of PBMCs.**

Parameter	Baseline
Sex (m/f)	4/6
Age (years)	25.9 ± 0.9
Body weight (kg)	62.7 ± 2.0
Height (m)	1.7 ± 0.0
BMI (kg/m ²)	21.7 ± 0.2
Fasting glucose (mg/dl)	91.1 ± 1.5
AST (U/L)	23.4 ± 2.2
ALT (U/L)	29.9 ± 4.7
Cholesterol (mg/dl)	165.3 ± 5.1
Triglycerides (mg/dl)	65.5 ± 9.7

542 Values are means ± standard error of means. ALT: alanine aminotransferase; AST: aspartate
 543 aminotransferase; BMI: body mass index; IAA, iso-alpha acids; LPS; lipopolysaccharide;
 544 PBMC: peripheral blood mononuclear cell.

545



Graphical abstract

546

547 **Graphical abstract:** Figure was created with BioRender.

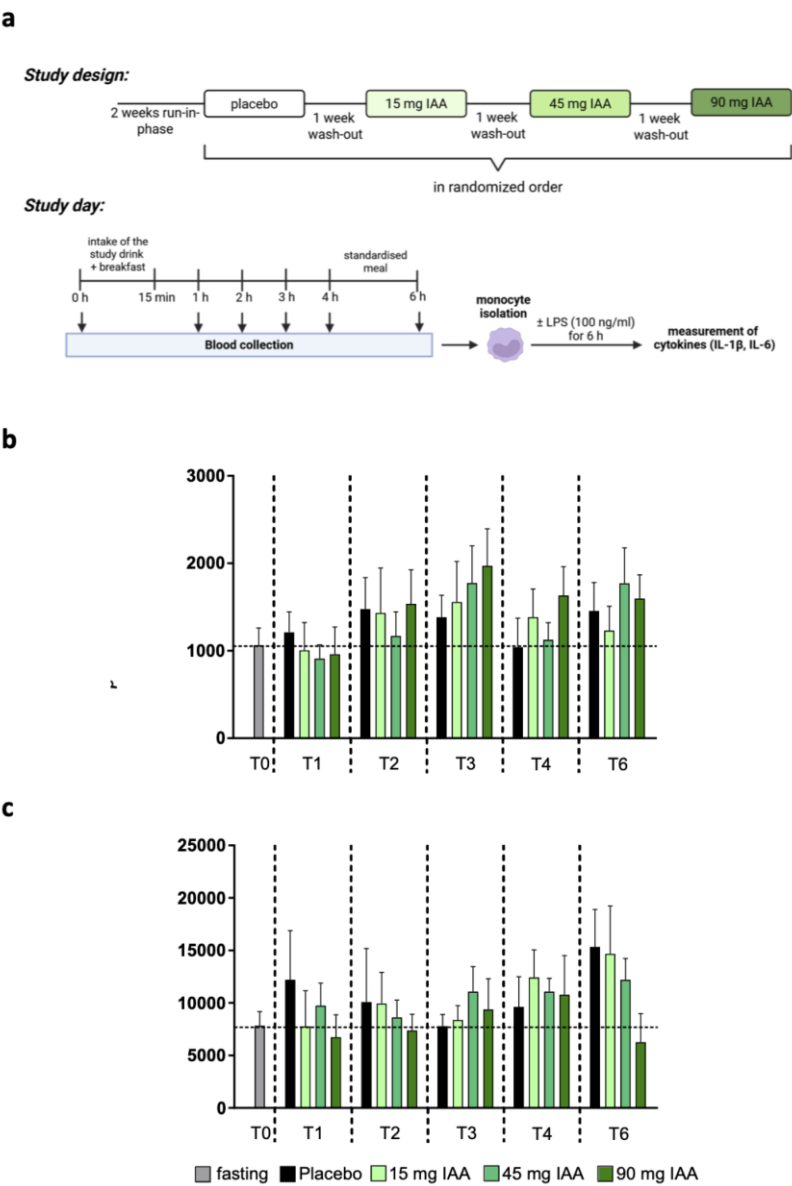


Figure 1

548

549 **Figure 1: Effect of a single intake of different concentrations of an iso-alpha acids-rich**
550 **hop extract on the LPS-induced immune response of IL-1β and IL-6 cytokine protein**

551 **concentration in monocytes of healthy female participants over time.** (a) Study design
552 and study day of the time- and dose-response study and protein concentration of (b) IL-1 β and
553 (c) IL-6 in cell culture supernatant of monocytes stimulated with 100 ng/ml LPS for 6 h isolated
554 from healthy female study participants of the time- and dose-response study receiving either a
555 placebo or IAA in different concentrations (15 mg, 45 mg, 90 mg). Data are presented as
556 means $\pm$ SEM, n = 5. The mean of IL-1 β and IL-6 protein concentration of LPS-stimulated
557 monocytes from fasting blood (T0) of all intervention groups are shown in one column. IAA,
558 iso-alpha acid; IL, interleukin; LPS, lipopolysaccharide.

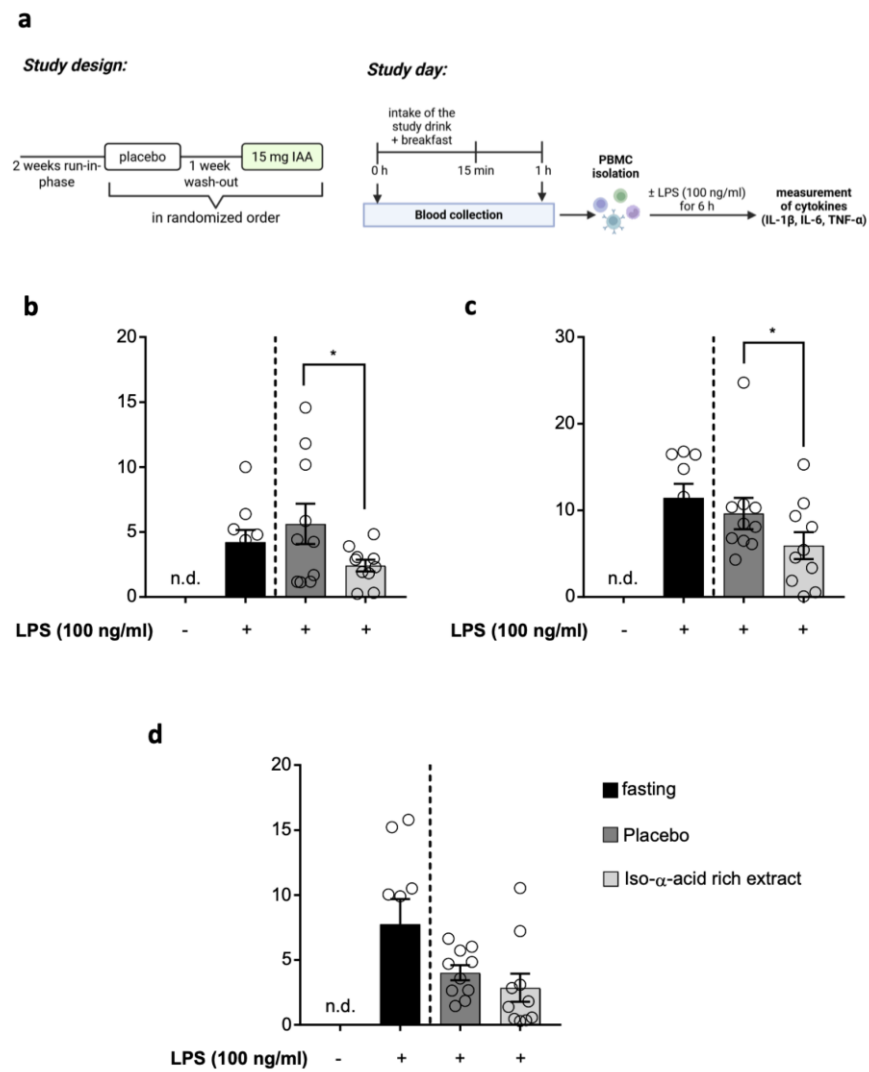


Figure 2

559

560 **Figure 2: Effect of a single intake of 15 mg of an iso-alpha acids-rich hop extract on the**
561 **LPS-induced immune response of proinflammatory cytokines in PBMCs of healthy**

30

562 **study participants.** (a) Study design and study day of the assessment of the acute effects of
563 IAA, protein concentrations of (b) IL-1 β , (c) IL-6 and (d) TNF- α in cell culture supernatant of
564 PBMCs stimulated with 0 or 100 ng/ml LPS for 6 h isolated from healthy study participants of
565 the assessment of the acute effects of IAA receiving either a placebo or 15 mg IAA. Data are
566 presented as means $\pm$ SEM, n = 10. *p < 0.05. IAA, iso-alpha acid; IL, interleukin; LPS,
567 lipopolysaccharide; n.d., not detectable = vehicle; PBMC, peripheral blood mononuclear cell;
568 TNF- α , tumour necrosis factor alpha.

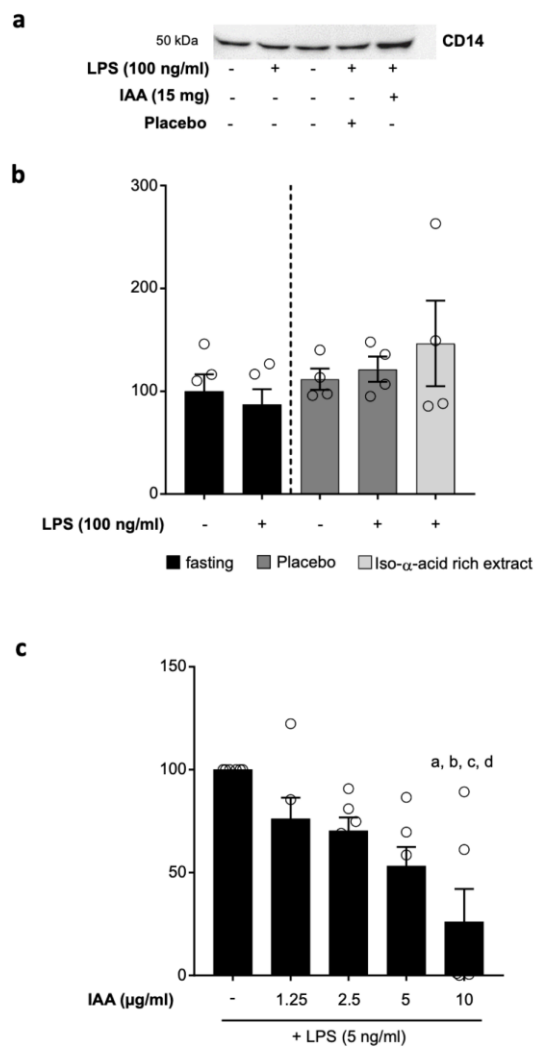


Figure 3

569

570 **Figure 3: Effect of iso-alpha acids-rich hop extract on the LPS-dependent CD14 protein**
571 **levels and TLR4 signaling.** (a) Representative blot and (b) densitometric analysis of CD14
572 western blot in cell culture supernatant of PBMCs obtained from study participants either
573 receiving a placebo or 15 mg of IAA stimulated with 100 ng/ml LPS for 24 h, (c) hTLR4 receptor

32

574 activity from HEK293 cells co-incubated with 5 ng/ml LPS and increasing concentrations of
575 IAA (0-10 µg/ml) for 12 h. Data are presented as means ± SEM, n= 5 - 6. ^ap < 0.05 compared
576 to LPS-stimulated cells, ^bp < 0.05 compared to LPS-stimulated cells treated with 1.25 µg/ml
577 IAA, ^cp < 0.05 compared to LPS-stimulated cells treated with 2.5 µg/ml IAA, ^dp < 0.05 compared
578 to LPS-stimulated cells treated with 5 µg/ml IAA. CD14, Cluster of Differentiation 14; HEK,
579 Human Embryonic Kidney (cells); IAA, iso-alpha acid; LPS, lipopolysaccharide; PBMC,
580 peripheral blood mononuclear cell.

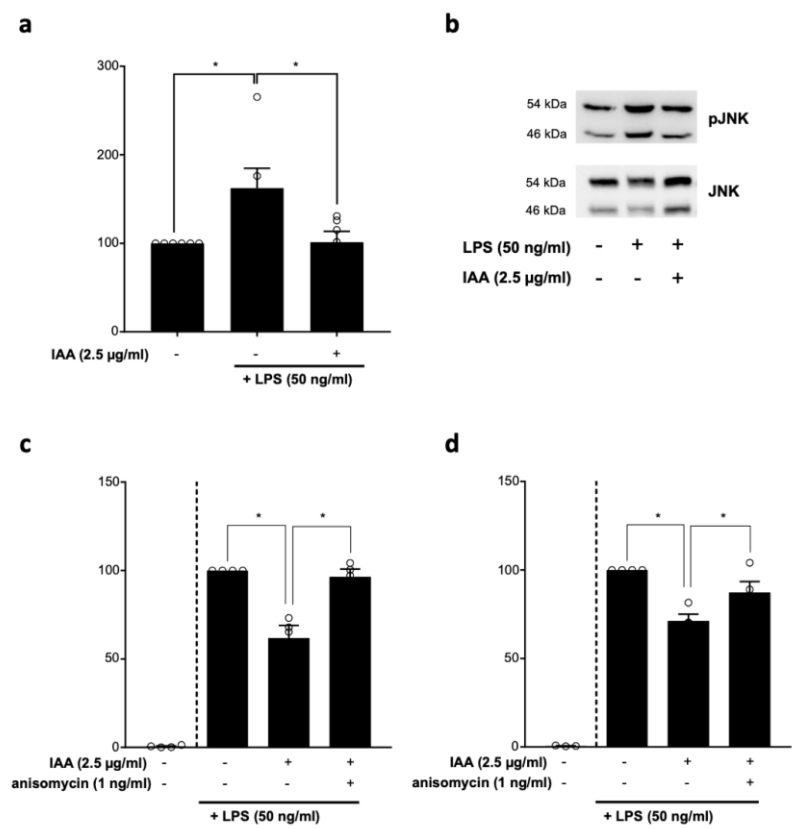


Figure 4

581

582 **Figure 4: Effect of an iso-alpha acids-rich hop extract and the MAPKinase agonist**
583 **anisomycin on the LPS-dependent activation of J774.A1 cells.** (a) Densitometric analysis
584 and (b) representative blot of phospho-JNK/JNK protein in J774A.1 cells stimulated with 0 or

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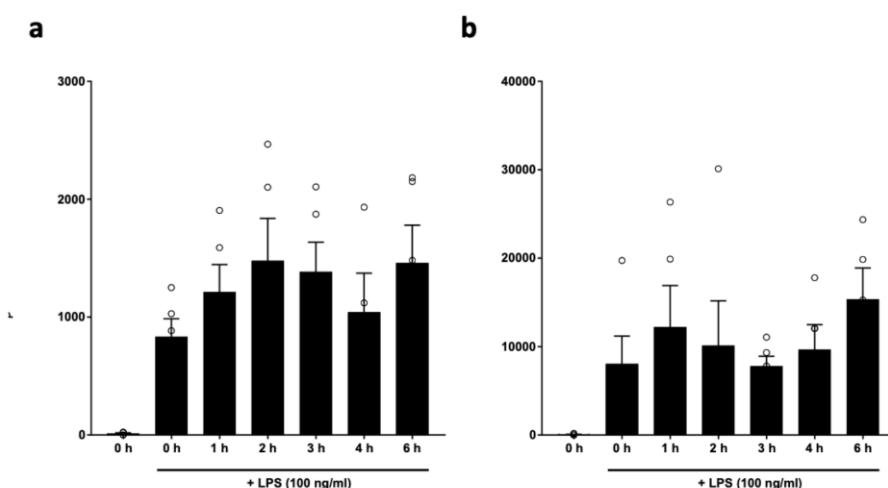
585 50 ng/ml LPS and 0 or 2.5 µg/ml IAA for 6 h, (c) protein concentration of TNF-α and (d) IL-6 in
586 cell culture supernatant of J774A.1 cells stimulated with 0 or 50 ng/ml LPS and 0 or 2.5 µg/ml
587 IAA and 0 or 1 ng/ml anisomycin for 6 h. Data are presented as means ± SEM, n = 4-6. *p <
588 0.05. IAA, iso-alpha acid; IL, interleukin; JNK, c-Jun N-terminal kinase; LPS,
589 lipopolysaccharide; TNF-α, tumour necrosis factor alpha.

590

Oral intake of low doses of an iso-alpha acid-rich hop extract dampens the LPS-mediated activation of blood immune cells: Randomized controlled trials in healthy individuals

Katja Csarman, Finn Jung, Anja Baumann, Benedikt Simbrunner, Kerstin Schweiger, Katharina Burger, Raphaela Staltner, Claus Hellerbrand, Ina Bergheim[#]

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Supplemental Figure 1: Effect of a 6 h incubation with LPS (100 ng/ml) on the IL-1 β and IL-6 release in monocytes isolated from healthy women before and 1 - 6 hours after the intake of the placebo. Protein concentration of (a) IL-1 β and (b) IL-6 in cell culture supernatant of monocytes stimulated with 100 ng/ml LPS for 6 h isolated from healthy female study participants of the time- and dose-response study receiving a placebo fasted and 1 - 6 h postprandial. Data are presented as means $\pm$ SEM, n = 4 - 5. IL, interleukin; LPS, lipopolysaccharide.

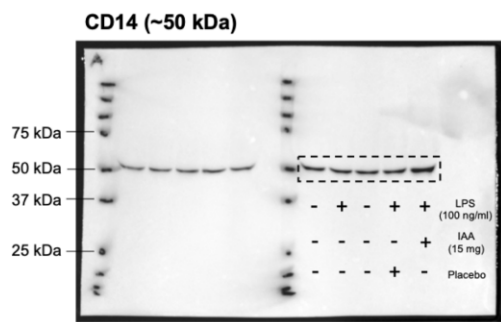
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Oral intake of low doses of an iso-alpha acid-rich hop extract dampens the LPS-mediated activation of blood immune cells: Randomized controlled trials in healthy individuals

Katja Csarmann, Finn Jung, Anja Baumann, Benedikt Simbrunner, Kerstin Schweiger, Katharina Burger, Raphaela Staltner, Claus Hellerbrand, Ina Bergheim[#]

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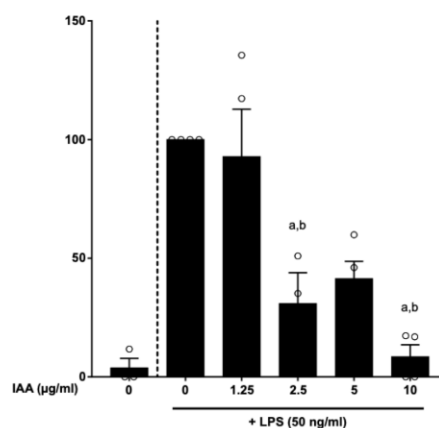


Supplemental Figure 2: Pictures of whole Western Blot of CD14 protein in cell culture supernatant of PBMCs obtained from study participants either receiving a placebo or 15 mg of IAA stimulated with 100 ng/ml LPS for 24 h. Bands that are shown at high magnification in Figure 3a are highlighted with a frame. CD14, Cluster of Differentiation 14; IAA, iso-alpha acid; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cell.

Oral intake of low doses of an iso-alpha acid-rich hop extract dampens the LPS-mediated activation of blood immune cells: Randomized controlled trials in healthy individuals

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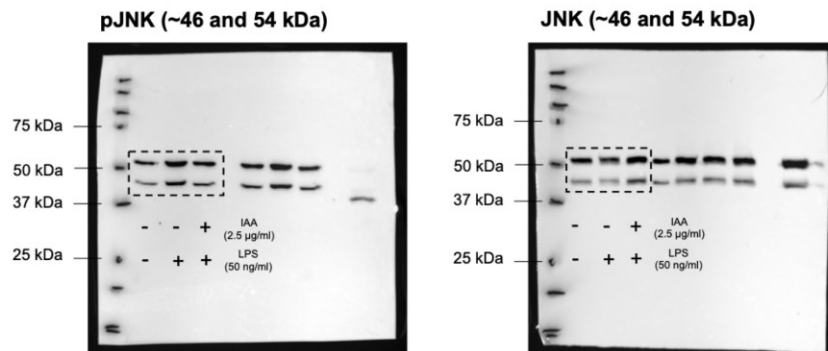


Supplemental Figure 3: Effect of different concentrations of an iso-alpha acids-rich hop extract on the LPS-dependent activation of J774A.1 cells. Protein concentration of IL-1 β in cell culture supernatant of J774A.1 cells stimulated with 0 or 50 ng/ml LPS and increasing concentrations of IAA-rich hop extract (0 – 10 μ g/ml) for 6 h. Data are presented as means $\pm$ SEM, n = 4. ^ap < 0.05 compared to LPS-stimulated cells, ^bp < 0.05 compared to LPS-stimulated cells treated with 1.25 μ g/ml IAA. IAA, iso-alpha acid; IL, interleukin; LPS, lipopolysaccharide.

Oral intake of low doses of an iso-alpha acid-rich hop extract dampens the LPS-mediated activation of blood immune cells: Randomized controlled trials in healthy individuals

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Supplemental Figure 4: Pictures of whole Western Blot of phospho-JNK/JNK protein in J774A.1 cells stimulated with 0 or 50 ng/ml LPS and 0 or 2.5 µg/ml IAA for 6 h. Bands that are shown at high magnification in Figure 4b are highlighted with a frame. IAA, iso-alpha acid; IL, interleukin; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide.

2.5. Unpublished data from a study entitled: A xanthohumol-rich hop extract as modulator of innate immunity and upper respiratory tract infection in adults. Publication 5

Katja Csarmann, Anja Baumann, Katharina Burger, Annette Brandt, Finn Jung, Benedikt Simbrunner, Kerstin Schweiger, Raphaela Staltner, Ina Bergheim.

Publication in preparation

Highlights:

- An acute oral intake of 0.75 mg xanthohumol through a xanthohumol-rich hop extract dampens the LPS-induced cytokine release (TNF- α , IL-6 and IL-1 β) for 1-6 hours in normal weight individuals.
- The 90-day long intake of xanthohumol (0.75 mg) twice daily was related with a significant decrease in symptom burden during cold episodes.
- Specifically, the duration of symptoms per cold infection and the area under the curve of symptom scores were significantly lower after xanthohumol ingestion in comparison to placebo.

Contribution to the manuscript:

- Planning parts of the study and contributing to preparing the ethics application
- Conducting parts of the human intervention study
- Conducting the *ex vivo* stimulation experiments
- Performing laboratory chemical analyses of human samples
- Analysing the data and performing statistical evaluations
- Preparing tables and figures

Total contribution: 20%

During the course of this dissertation, several human intervention studies were conducted; however, for some study parameters, data analysis was not completed in time to allow inclusion in this thesis in the form of a manuscript.

2.5.1. Methods used to acquire the so far unpublished data

Human intervention studies

All intervention studies were carried out in a randomized placebo-controlled design and in accordance with the ethical standards laid down in the Declaration of Helsinki of 1975, as revised in 1983. All studies were approved by the ethics committee of the University of Vienna, Vienna, Austria (reference numbers: 00848, 00900 and 00586) and were registered at ClinicalTrials.gov (NCT06286644 and NCT06286657) and conducted in accordance with the Consolidated Standards of Reporting Trials (CONSORT) guidelines for randomized clinical trials¹²⁷. The following exclusion criteria were defined for the studies: (1) following a special diet or (2) food malabsorption or (3) a history of diseases of the gastrointestinal tract. Only non-smoking individuals were enrolled after providing written informed consent and were required not to have had a viral or bacterial infection within 3 weeks prior to the study. Furthermore, the use of anti-inflammatory medication was defined as an exclusion criterion for the studies. In all three studies, participants were asked to refrain from consuming hop products one week prior to the studies and during the intervention.

Time- and dose- response study in normal-weight volunteers: To determine the optimal dose of xanthohumol (XN) to influence the immune response after the ingestion of the XN-rich hop extract, a single-blinded, randomized pilot study was conducted in seven normal-weight (BMI: 18.5-24.9 kg/m²), healthy volunteers. To prepare the study drink, 0, 0.125, 0.375 or 0.75 mg XN of the XN-containing extract (Xantho-Flav, Hopsteiner GmbH, Germany) was mixed with powdered-sugar, citric acid and a binding agent and was freshly dissolved in 50 ml of water. Drinks were prepared right after an overnight fast and obtaining a blood sample, subjects were

asked to consume the study drink within 15 minutes together with a standardised breakfast (2 medium-sized pretzels with 30 g butter). 1, 2, 3, 4 and 6 hours after the consumption of the study drink blood was taken to isolate monocytes (see below). Between 4 and 6 hours after the intake of the study drink, all participants consumed a second standardised meal. After the first study day and a 1-week wash-out phase, in which the participants again were asked to refrain from all hop-containing foods and beverages, the intervention was repeated in a cross-over design until all concentrations of xanthohumol had been consumed. The study design and the procedure on the study day are summarized in Figure 1a. Clinical parameters were determined in the serum and monocytes were isolated from the whole blood as detailed below. For basic characteristics of the study subjects, see Table 1.

Long-term study in normal- and overweight volunteers: Based on the time- and dose-response study, 0.75 mg xanthohumol was selected to assess the effect of a twice-daily intake, compared to placebo, on upper respiratory infections. 39 normal-weight and overweight (BMI: 18.5-29.9 kg/m²) women (age 21-49 years) were randomly assigned and single-blinded to receive a study drink as described above, containing 0 or 0.75 mg of xanthohumol derived from a xanthohumol-rich hop extract. The study drink was consumed twice daily for 90 days. On the first study day, on day 45, and after 90 days, participants underwent a fasting blood draw, and anthropometric measurements were taken. During the 90-day intervention, study participants completed a health diary and recorded symptoms of illness. As soon as at least two symptoms (listed as: runny nose, blocked nose, sore throat, coughing, sneezing, coloured discharge and flu-like symptoms (fever, headache, general aches and pains, fatigue and weakness or chest discomfort)) occurred on two consecutive days, the Wisconsin Upper Respiratory Symptom Survey (WURSS-24)¹²⁸,

a validated illness-specific questionnaire assessing symptom severity and quality-of-life impact, was completed by participants until recovery. Items were rated on a 7-point Likert-style severity scale. For each participant, a daily total global severity score was calculated by summing WURSS-24 items 2–23. Furthermore, participants were asked to perform a rapid antigen test capable of detecting SARS-CoV-2, influenza and respiratory syncytial virus (RSV), in order to determine the type of respiratory pathogen involved. The study design and the procedure on the study day are summarized in Figure 2a. Clinical parameters were determined in the serum. For basic characteristics of the study subjects, see Table 2.

Isolation of monocytes from the time- and dose- response study

Monocytes were isolated from whole blood samples collected from participants in the time- and dose-response study. Monocytes were isolated from whole blood samples as detailed previously¹²⁹ using the pluriSelect system following the instructions of the manufacturer (pluriSelect Life Science UG & Co. KG, Leipzig, Germany). In brief, blood was layered onto monocyte density gradient media (1.068 g/ml) in separation tubes (pluriSelect Life Science UG & Co. KG, Leipzig, Germany) and centrifuged at room temperature for 15 minutes. Cells were collected and used for further experiments as described below.

Isolation of monocytes from naïve individuals

Monocytes were isolated from whole blood samples collected from healthy, naïve donors. After obtaining written informed consent and following an overnight fast, blood was drawn from healthy, normal weight donors, with approval from the ethics committee of the University of Vienna, Vienna, Austria (reference number 00586). Monocytes were isolated from whole blood samples as detailed previously¹²⁹ using the pluriSelect system following the instructions of the manufacturer (pluriSelect Life

Science UG & Co. KG, Leipzig, Germany). In brief, blood was layered onto monocyte density gradient media (1.068 g/ml) in separation tubes (pluriSelect Life Science UG & Co. KG, Leipzig, Germany) and centrifuged at room temperature for 15 minutes. Cells were collected and used for further experiments as described below.

Stimulation of monocytes from the time- and dose- response study

Isolated monocytes were cultivated in RPMI-1640 medium (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) with 10% fetal bovine serum (Pan-Biotech GmbH, Aidenbach Germany) and 100 µg/ml streptomycin and 100 U/ml penicillin (Pan-Biotech GmbH, Aidenbach, Germany) at 37 °C in a humidified 5% CO₂ atmosphere for 1 h. Subsequently, cells were challenged with 100 ng/ml LPS for 6 h respectively. Cell culture supernatant was collected at the end of the experiment and stored at –80 °C until further use.

Stimulation of monocytes obtained from naïve healthy donors

Isolated monocytes from eight naïve healthy donors were cultivated in RPMI-1640 medium (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) with 10% fetal bovine serum (Pan-Biotech GmbH, Aidenbach Germany) and 100 µg/ml streptomycin and 100 U/ml penicillin (Pan-Biotech GmbH, Aidenbach, Germany) at 37 °C in a humidified 5% CO₂ atmosphere for 1 h. Subsequently, cells were challenged with or without 10 ng/ml LPS with or without soluble CD14 (500 ng/ml) (Thermo Fisher Scientific, Massachusetts, USA) and with or without xanthohumol (8 µg/ml) (Xantho-Flav, Hopsteiner GmbH, Germany) for 6 h respectively. Cell culture supernatant was collected at the end of the experiment and stored at –80 °C until further use.

Enzyme-Linked Immunosorbent Assays (ELISA's)

IL-1 β , IL-6 and TNF- α protein concentrations were analysed in cell culture supernatant of monocytes using commercially available DuoSet® ELISA Development Systems (R&D®, Minneapolis, USA) kits.

Statistical Analyses

Data are presented as means $\pm$ standard error of the means (SEMs). Grubb's test was performed before statistical analysis to identify outliers. Homogeneity of variances was tested and data were log-transformed if they were not normally distributed or in case of inhomogeneity of variances before performing further statistical tests. A t-test was used to determine statistically significant differences between two groups and one-way ANOVA was used for all other comparisons (GraphPad Prism Software, CA, USA). A p-value ≤ 0.05 was considered statistically significant.

2.5.2. Results

LPS-stimulation of monocytes isolated from healthy adults 1-6 h after the intake of 0-0.75 mg XN through a XN-rich hop extract

In a randomized placebo-controlled single-blinded cross-over human intervention study the effect of an acute, one-time intake of a beverage containing 0, 0.125, 0.375 and 0.75 mg XN derived through a XN-rich hop extract on LPS-dependent cytokine release was assessed in monocytes isolated from seven healthy, normal weight women and men (characteristics see Table 1). When comparing cytokine protein levels in cell culture medium of cells isolated after the intake of the placebo with those detected after the intake of the different XN-rich drinks we observed that IL-1 β concentrations were lower following intake of all three xanthohumol concentrations, except for 0.125 mg after 2 h. A similar trend was observed for IL-6, where XN intake resulted in a modest reduction in the release of the cytokine compared to placebo at several time points. However, the effect was less consistent for 0.375 mg XN after 2 h and 4 h. The release of TNF- α was consistently lower for all XN doses after 1 h and, thereafter, at several time points compared to placebo intake. However, the effect was less consistent for 0.75 mg XN after 2 h and 4 h (see Figure 1 b-d).

Based on the preliminary study with normal weight subjects, the beverage containing 0.75 mg XN was selected for the 90-day intervention study.

Table 1. Characteristics of healthy subjects enrolled in the time- and dose response study.

Parameter	Baseline
Sex (m/f)	3/4
Age (years)	25.3 ± 0.6
Body weight (kg)	67.5 ± 3.6
Height (m)	1.7 ± 0.0
BMI (kg/m ²)	22.4 ± 0.7
Fasting glucose (mg/dl)	76.2 ± 4.1
AST (U/L)	24.8 ± 4.2
ALT (U/L)	26.3 ± 5.5
Cholesterol (mg/dl)	163.9 ± 8.0
Triglycerides (mg/dl)	92.6 ± 16.1

Values are means ± standard error of means. ALT: alanine aminotransferase; AST: aspartate aminotransferase; BMI: body mass index.

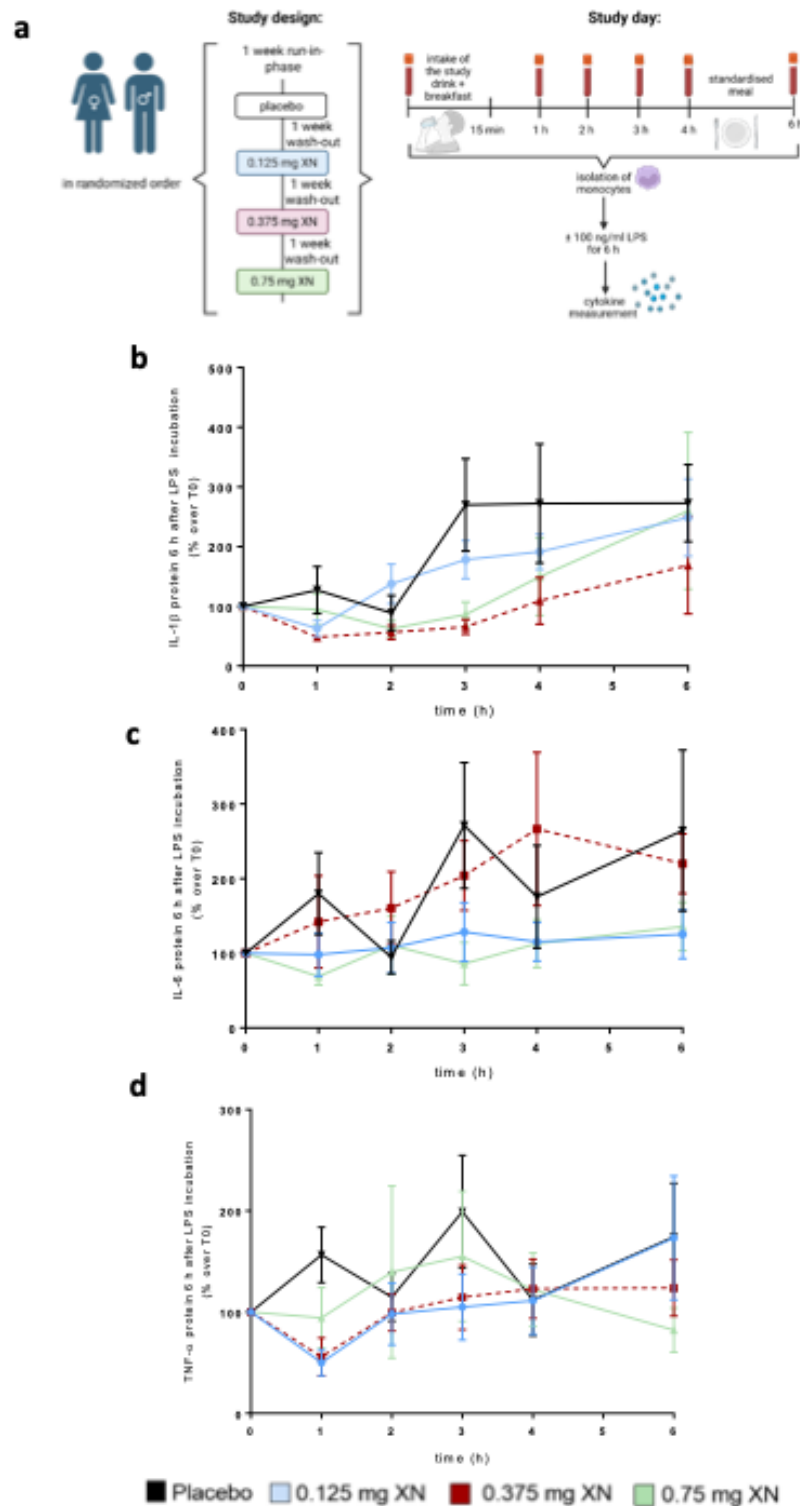


Figure 1

Figure 1: Effect of a single intake of different concentrations of a xanthohumol-rich hop extract or placebo on the LPS-induced immune response of pro-inflammatory cytokine release in monocytes of healthy normal weight participants over time. (a) Study design and study day of the time- and dose- response

study, protein concentrations of **(b)** IL-1 β , **(c)** IL-6 and **(d)** TNF- α over 6 h in cell culture supernatant of monocytes stimulated with 100 ng/ml LPS for 6 h isolated from healthy study participants of the time- and dose- response study receiving either a placebo or XN in different concentrations (0 mg, 0.125 mg, 0.375 mg, 0.75 mg). Data are presented as means $\pm$ SEM. n=7 (except for XN doses 0.125 and 0.375 mg n=6). IL interleukin, LPS lipopolysaccharide, XN xanthohumol.

Upper respiratory tract infections during a 90-day intervention with 0.75 mg XN through a XN-rich hop extract in normal-weight and overweight women

In a second placebo-controlled, randomized intervention study, the effect of consuming a beverage containing 0.75 mg of XN derived from a XN-rich hop extract twice daily for 90 days on the duration, severity and prevalence of upper respiratory tract infections was assessed (see Figure 2a for the study design and Table 2 for the baseline characteristics of the study participants). In total, 42 participants initially enrolled in the study; however, drop-outs occurred (n=2 in the placebo and n=1 in the XN group) due to illness/discomfort, resulting in 39 participants included in the final analysis (Placebo group n=19, XN group n=20). The groups were similar with respect to age, BMI, seasonal influenza and COVID-19 vaccination as well as clinical parameters. No adverse events related to the study drinks were reported. Furthermore, general health markers remained unchanged, except for diastolic blood pressure, which was significantly lower in both groups at the end of the study after 90 days compared to baseline (see Table 3). Participants who exhibited cold or flu symptoms on two consecutive days were required to complete the WURSS-24 questionnaire, a validated illness-specific questionnaire assessing symptom severity and quality-of-life impact. During the 90-day intervention, 8 women in the placebo group and 13 women in the XN group experienced at least one upper respiratory tract infection (URTI) episode, corresponding to an incidence of 42.1 % and 65.0 %, respectively (see Table 4 for participant characteristics). As assessed through a rapid antigen test, none of the participants in the XN group tested positive for SARS-CoV-2, influenza or RSV, while in the placebo group, two participants tested positive, one for influenza infection and one for RSV.

When analysing the WURSS-24 outcomes, which are based on responses recorded on a Likert scale and subsequently used to calculate composite scores over the course of each illness episode, the area under the curve (AUC) for the total score (combining functional and symptom scores), as well as the function score (reflecting the impact of illness on daily activities), were comparable between groups (see Figure 2b,c). The AUC for the symptom score (including nasal, throat, and systemic symptoms) was significantly lower in the XN group compared to placebo (see Figure 2d). The AUC for subjective illness severity, reflecting the participants' overall perception of illness, tended to be lower in the XN group compared to placebo ($p=0.0737$) (see Figure 2e). The duration of symptoms per episode was significantly shorter in the XN group (3 days) compared to the placebo group (4 days) (see Table 4).

Table 2. Baseline characteristics of healthy subjects enrolled in a study to assess the long-term effects of xanthohumol on the immune response.

Parameter	Placebo	Xanthohumol
Sex (m/f)	0/19	0/20
Age (years)	26.2 ± 1.5	26.3 ± 1.4
Body weight (kg)	65.0 ± 2.0	61.9 ± 1.8
Height (m)	1.7 ± 0.0	1.7 ± 0.0
BMI (kg/m ²)	23.1 ± 0.6	22.0 ± 0.6
Seasonal Covid-19 vaccination (%)	15	25
Seasonal Influenza vaccination (%)	5	5

Values are means ± standard error of means. BMI: body mass index.

Table 3. Long-term (90 d) effects of xanthohumol on the immune response.

Parameter	Placebo T0	Placebo T90	Xanthohumol T0	Xanthohumol T90
Systolic blood pressure (mmHg)	117.2 ± 1.9	118.5 ± 2.1	125.7 ± 2.2	122.2 ± 2.0
Diastolic blood pressure (mmHg)	79.3 ± 1.5	76.2 ± 1.7*	84.3 ± 1.6	80.9 ± 1.7*
Fasting glucose (mg/dl)	84.2 ± 1.7	81.2 ± 1.7	85.3 ± 2.0	84.7 ± 1.8
AST (U/L)	22.7 ± 1.2	21.5 ± 1.0	20.8 ± 1.6	21.5 ± 0.7
ALT (U/L)	18.8 ± 1.4	19.2 ± 1.4	16.4 ± 1.5	16.5 ± 1.0
Cholesterol (mg/dl)	173.8 ± 6.0	171.3 ± 8.0	170.9 ± 7.8	166.7 ± 7.4
Triglycerides (mg/dl)	76.3 ± 5.0	70.8 ± 5.4	69.5.0 ± 5.5	73.4 ± 5.3

Values are means ± standard error of means. ALT: alanine aminotransferase; AST: aspartate aminotransferase. *p≤0.05 compared to T0.

Table 4. WURSS-24 analysis of healthy subjects enrolled in a study to assess the long-term effects of xanthohumol on the immune response.

Parameter	Placebo n=19	Xanthohumol n=20	p-value
Individuals with URTI	8	13	0.205
Incidence (%)	42.1	65.0	0.205
Nr. of episodes	1	1	n.s.
Duration of symptoms per episode (d)	4	3*	*0.0495

Values are means ± standard error of means. URTI: upper respiratory tract infection. *p≤0.05 compared to placebo.

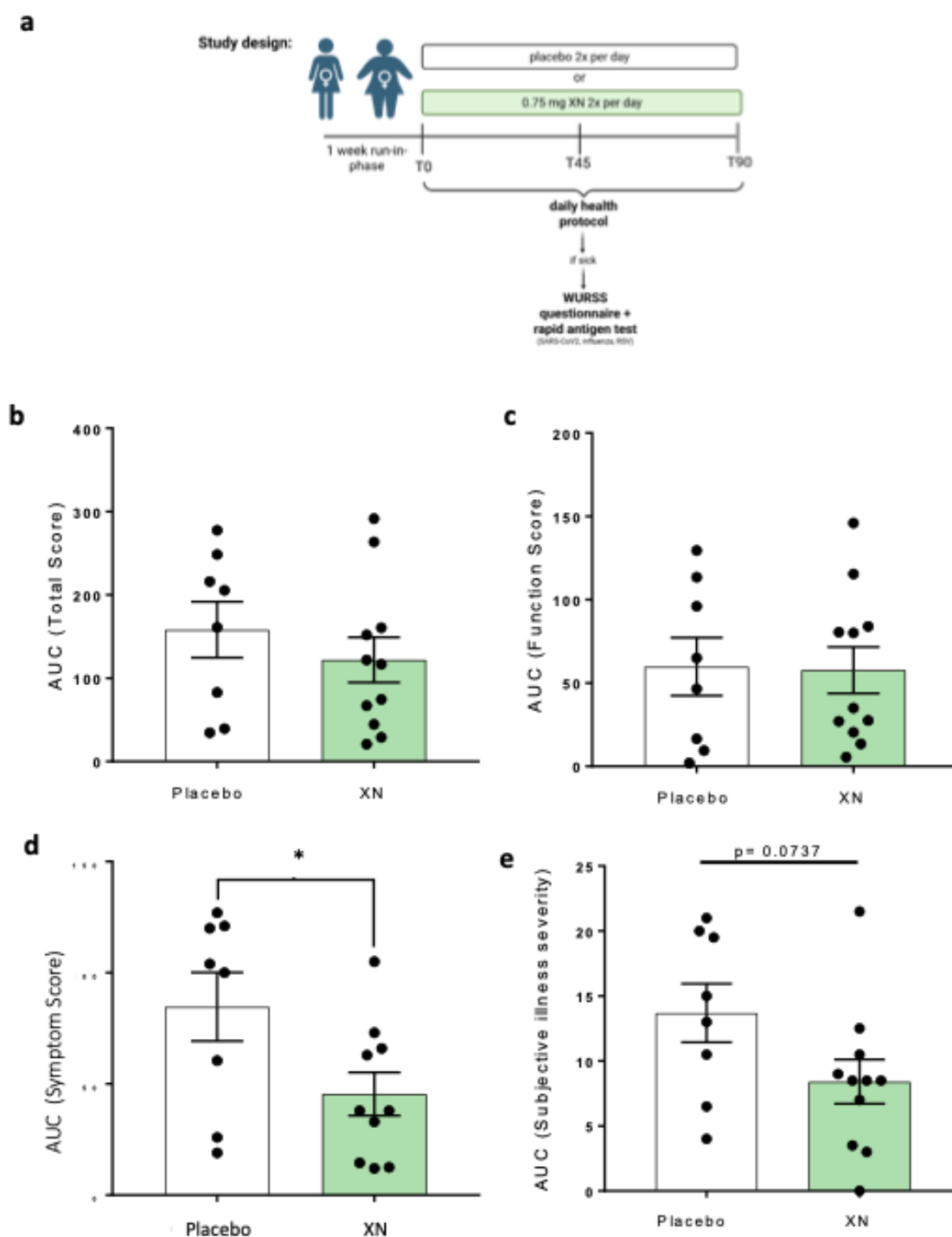


Figure 2

Figure 2: Effect of a daily consumption of 2 x 0.75 mg xanthohumol-rich hop extract or a placebo over 90 days on health parameters like susceptibility to diseases, severity and symptoms. (a) Study design and study day of the assessment of the long-term consumption of a xanthohumol rich hop extract, AUC of the

(b) total score, (c) function score (impairment in everyday life), (d) symptom score (severity of symptoms), and (e) subjective feeling of illness in people that got sick.

Data are presented as means $\pm$ SEM. n = 8 - 11. * $p \leq 0.05$. AUC Area under the curve, WURSS Wisconsin Upper Respiratory Symptom Survey, XN xanthohumol.

To further investigate the molecular mechanism, naïve primary monocytes were incubated *ex vivo* with xanthohumol and soluble CD14 (sCD14) and subsequently stimulated with 10 ng/ml LPS, which was selected to mimic a milder infectious stimulus and to better determine the effects of XN on IL-1 β release. The presence of sCD14 significantly increased IL-1 β secretion in LPS-stimulated cells, whereas concomitant treatment with XN significantly reduced IL-1 β levels (see Figure 3).

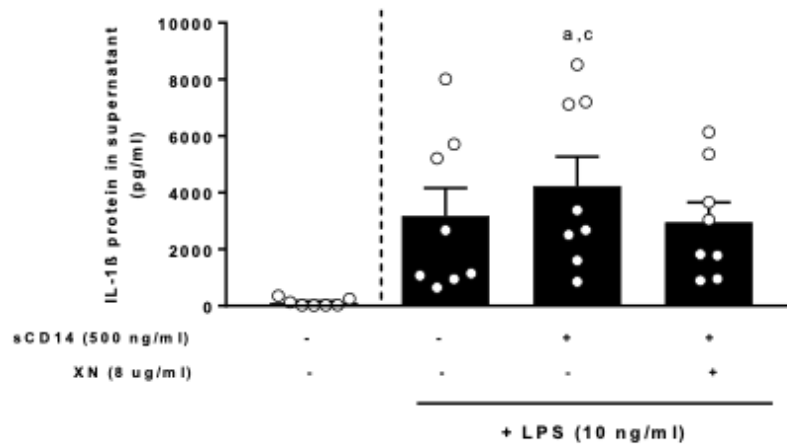


Figure 3

Figure 3: Interaction of sCD14 and xanthohumol in the regulation of LPS-induced IL-1 β protein secretion in human monocytes. IL-1 β protein concentration in cell culture supernatant of naïve monocytes that were challenged with or without sCD14 (500 ng/ml), with or without XN (8 μ g/ml) and with LPS (10 ng/ml) over 6 h. Data are presented as means $\pm$ SEM. $n = 8$. ^a $p \leq 0.05$ compared to LPS (10 ng/ml) stimulation, ^c $p \leq 0.05$ compared to LPS (10 ng/ml) with sCD14 (500 ng/ml) with XN (8 μ g/ml) stimulation. sCD14 soluble cluster of differentiation 14, IL interleukin, LPS lipopolysaccharide, XN xanthohumol.

3. Discussion

Diet plays a key role in maintaining health and regulating disease processes. Diet and compounds consumed through different foods play a critical role in maintaining intestinal barrier integrity, limiting the translocation of bacteria and their toxins while enabling nutrient absorption¹³⁰. The present dissertation aimed to elucidate how dietary factors such as fructose can modulate intestinal barrier function and innate immune responses, thereby contributing to inflammatory processes relevant to metabolic and infectious diseases. In addition, the potential of hop-derived compounds to modulate innate immune signalling and inflammatory responses in humans was investigated.

3.1. Dietary fructose as a modulator of intestinal barrier function and innate immune responsiveness

Genetic predisposition and low physical activity, as well as an unhealthy diet rich in saturated fatty acids and sugars, particularly fructose, are associated with the development of MASLD (for an overview, see¹³¹). In this context, disturbances of intestinal homeostasis, including tight junction disruption, increased intestinal permeability, loss of the mucus layer, and dysbiosis, have been identified as central mechanisms contributing to bacterial translocation and hepatic inflammation in MASLD¹³².

The results of this dissertation suggest that fructose, even in the absence of intestinal microbiota, impairs intestinal barrier integrity through NO-mediated tight junction disruption.

In line with our findings, previous studies from our group and others have demonstrated that broad-spectrum antibiotic treatment diminishes MASLD development in rodent models, underscoring the relevance of intestinal barrier integrity in disease pathogenesis¹³³⁻¹³⁵. Here we further demonstrated that fructose appears to contribute to metabolic diseases not only through its caloric load, but also via direct effects on intestinal barrier function. Supporting this concept, a study by Guo et al. in piglets suggests that fructose-associated barrier alterations may not only result from changes in intestinal microbiota composition, but may also arise from direct effects of fructose metabolism in enterocytes, as chronic fructose intake was shown to decrease the expression of tight junction proteins¹³⁶. Consistent with these findings, the present dissertation further adds weight to the hypothesis that high fructose intake may directly alter intestinal barrier function, as evidenced by increased permeability and altered tight junction regulation (see Publication 1). Furthermore, elevated relative iNOS protein levels were observed in mice fed a high fructose diet (see Publication 1). In line with this, previous reports demonstrate that genetic or pharmacological inhibition of iNOS abolishes LPS-induced bacterial translocation and intestinal barrier damage^{137,138}, underscoring the central role of NO-dependent mechanisms in maintaining barrier integrity.

Since impaired intestinal barrier integrity increases systemic exposure to bacterial ligands, an important question is whether fructose not only promotes ligand translocation but also alters host responsiveness to such stimuli.

Results of this dissertation suggest that fructose intake metabolically and SP1-dependently upregulates TLR2 expression in human monocytes, thereby amplifying TLR2-driven pro-inflammatory cytokine responses.

However, as this study used an *ex vivo* approach with postprandial stimulation of immune cells, it remains unclear whether these findings translate to humans in the presence of an active infection.

From a nutritional perspective, it is important to consider both the type of sugar consumed and the matrix in which fructose is ingested. Epidemiological studies indicate that in many regions worldwide, a substantial proportion of dietary sugar intake derives from sugar-sweetened beverages, with average sugar consumption ranging around 100 g per day¹³⁹⁻¹⁴¹. Furthermore, Staltner et al. demonstrated that the intake of a beverage containing moderate amounts of fructose (58 g/l) is sufficient to induce postprandial translocation of bacterial endotoxin and TLR2 ligands into the circulation of healthy individuals¹⁴². Notably, no comparable increase in circulating endotoxin levels was observed when an isocaloric, naturally cloudy apple juice spritzer was consumed or when fructose was replaced by non-fructose carbohydrates, such as maltodextrin, or artificial sweeteners, such as sucralose¹⁴². These findings suggest that fructose-induced barrier dysfunction may critically depend on the matrix in which fructose is consumed, rather than sugar content per se. In this context, matrix-associated components present in fruit-based beverages may partially counteract fructose-induced intestinal barrier disruption.

While alterations of the intestinal microbiota have been proposed as a contributing factor, sugar-sweetened beverage consumption has also been associated with dysbiosis in humans^{140,143,144}. Recent human and animal studies further indicate that fructose may directly affect intestinal NO homeostasis and tight junction integrity in the small intestine^{77,86,145}. However, conflicting evidence exists, as short-term fructose consumption in humans did not uniformly result in alterations of intestinal

permeability or microbiota composition¹⁴⁶. The precise molecular mechanisms underlying these context-dependent effects of fructose therefore remain to be elucidated.

Previous studies have shown that fructose promotes the development of a pro-inflammatory immune phenotype¹⁴⁷⁻¹⁵⁰, yet the underlying molecular mechanisms remain incompletely understood. In the present work, we demonstrated in humans that TLR2 upregulation following fructose intake increased monocyte responsiveness to *ex vivo* LTA stimulation, but not maltodextrin. Consistent with this, Jones et al. reported enhanced cytokine induction in monocytes following fructose incubation and subsequent LPS stimulation¹⁵⁰.

Taken together, these findings suggest that fructose-induced metabolic stress alters hepatic energy homeostasis and primes innate immune signalling, thereby linking metabolic and inflammatory pathways and supporting the idea that dietary fructose is a relevant and potentially modifiable factor in intestinal barrier dysfunction and metabolic inflammation.

3.2. Hop-derived compounds as nutritional modulators of Toll-like receptor-dependent inflammatory signalling

After investigating fructose as a dietary trigger contributing to metabolic disease onset, intestinal barrier dysfunction and immune dysregulation, the present dissertation next addressed the potential of nutritionally relevant bioactive compounds to modulate innate immune responses. In this context, the hop-derived bitter acid IAA and the flavonoid xanthohumol were investigated as dietary modulators of inflammation in humans. Previous studies in rodents and humans have demonstrated beneficial metabolic effects of IAA on risk factors associated with metabolic syndrome, including modulation of blood lipid profiles¹⁵¹, reduction of endotoxemia¹⁵²,

improvement of insulin resistance¹⁵³ and attenuation of MASLD¹⁰⁷. These metabolic disorders are closely linked to chronic low-grade inflammation and nutritional strategies employing anti-inflammatory bioactives such as IAA may represent promising approaches to dampen excessive innate immune activation¹⁵⁴⁻¹⁵⁶.

The present dissertation demonstrates that acute oral intake of physiologically relevant doses of IAA through a hop-rich extract suppresses pro-inflammatory cytokine release in response to bacterial ligands in healthy individuals.

While previous murine studies mainly reported anti-inflammatory effects of IAA in neuroinflammatory settings or models of alcohol-and metabolism-associated liver injury^{104,107,157,158}, the present findings suggest that IAA can modulate innate immune responses at nutritionally relevant doses, which were generally lower than those applied in previous murine studies.

A central mechanistic finding of this work is that IAA primarily modulates intracellular signalling downstream of receptor activation rather than affecting PRR abundance or ligand recognition. Accordingly, neither IAA intake nor LPS stimulation altered CD14 protein levels. However, IAA selectively attenuated LPS-induced TLR4 activation (see Publication 4), while leaving LTA-induced TLR2 signalling unaffected (see Publication 3). This differential modulation is consistent with differences between TLR pathways¹⁵⁹. Whereas TLR2 forms heterodimers with TLR1 or TLR6 and signals predominantly via MyD88/TIRAP, TLR4 recognizes LPS from the cell wall of Gram-negative bacteria and activates MyD88-dependent pathways, resulting in qualitative differences in signal integration and downstream inflammatory responses¹⁵⁹. These pathway-specific characteristics likely underlie the selective

inhibition of TLR signalling observed for IAA. Similarly, polyphenolic bioactive compounds such as epigallocatechin-3-gallate and quercetin have been shown to selectively attenuate TLR4-mediated inflammatory responses by modulating intracellular signalling cascades downstream of receptor activation rather than altering PRRs expression or ligand recognition^{160,161}.

Although receptor-level effects appeared more selective for TLR4, downstream signalling suppression in J774A.1 cells was observed irrespective of upstream receptor activation, suggesting that IAA may converge on shared inflammatory signalling nodes such as JNK and p38. Differences from previous studies describing no effects of related hop-derived compounds on p38 or JNK phosphorylation in RAW 264.7 cells¹⁶² may reflect differences in compound composition (IAA versus rho iso- α acids), species-specific signalling characteristics, or experimental model systems.

Complementing these acute, mechanistically focused studies with IAA, the present dissertation also investigated xanthohumol as a hop-derived bioactive compound, allowing evaluation of both acute and 90-day intervention effects on innate immune function (see Publication 5).

In line with previous studies, the present work demonstrates that both acute and long-term intake of xanthohumol may dampen LPS-dependent immune responses in human monocytes and may reduce cold symptoms, duration and severity.

Mechanistic support for these observations is provided by two additional studies from our group demonstrating that low doses of xanthohumol acutely modulate TLR-dependent immune responses in humans. Jung et al. further demonstrated that oral intake of xanthohumol attenuated both LTA- and LPS-induced cytokine release in

PBMCs and suppressed TLR2- and TLR4-mediated signalling^{122,123}. Moreover, independent of the present findings, xanthohumol and its prenylated derivatives have been reported to interfere with viral protease activity, including that of SARS-CoV-2, indicating that these compounds may exert both host-directed immunomodulatory and pathogen-directed effects¹⁶³.

These observations suggest that long-term xanthohumol intake may not prevent infection per se but may attenuate the severity and duration of infection-associated inflammatory responses through combined host-directed immunomodulatory and pathogen-directed mechanisms. Taken together, the results of this dissertation together with studies from Jung et al. suggest that intake of IAA (15 mg) and xanthohumol (0.75 mg) may represent novel approaches for modulating inflammatory responses in human immune cells, likely through modulation of downstream signalling pathways such as JNK or p38 (see Figure 6)^{122,123}. In contrast to fructose, which appears to promote innate immune activation, hop-derived bitter compounds may counteract excessive inflammatory signalling, highlighting nutrition as both a driver and modulator of immune function.

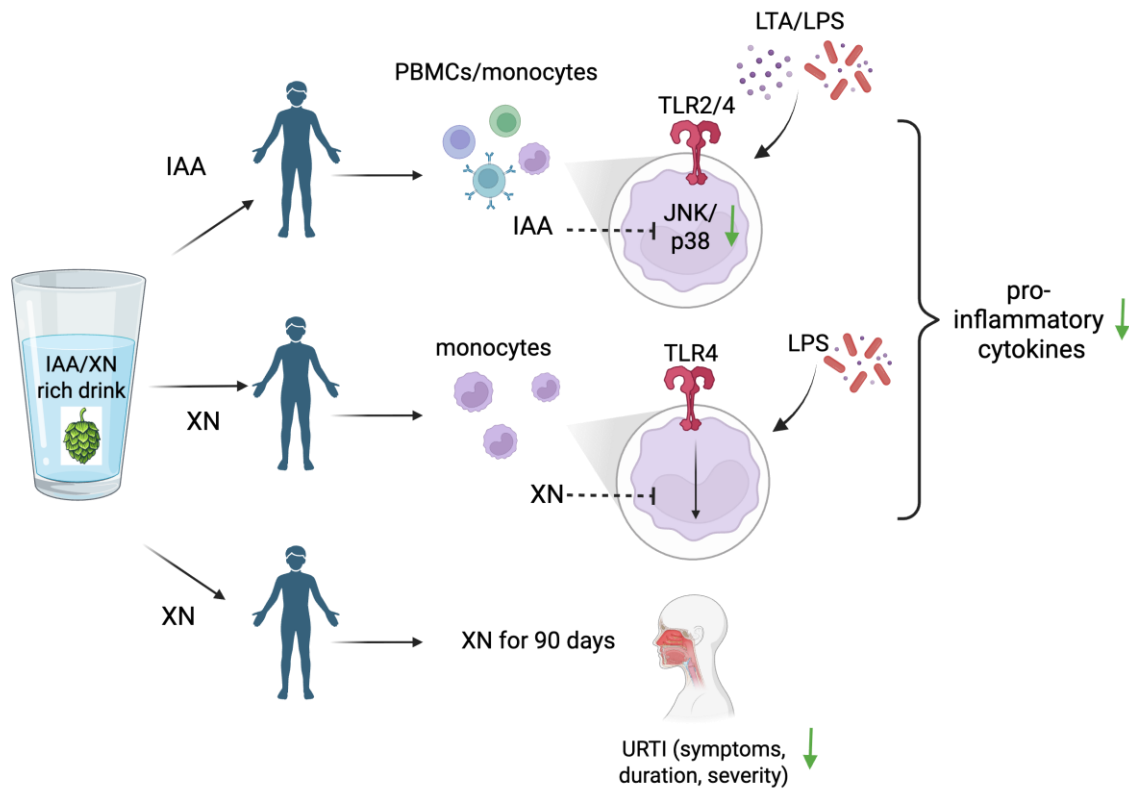


Figure 6: Graphical summary of hop-derived compounds as modulators of Toll-like receptor-dependent inflammatory signalling and upper respiratory tract infections

The findings of this dissertation indicate that 1) ingestion of an IAA-rich hop extract attenuate cytokine responses through JNK/p38-dependent pathways, and 2) long-term ingestion of an XN-rich hop extract reduces URTI severity. IAA: Iso-α acids; JNK: C-jun-N-terminal kinase; LPS: Lipopolysaccharide; LTA: Lipoteichoic acid; PBMCs: Peripheral blood mononuclear cells; TLR: Toll-like receptor 2/4; URTI: upper respiratory tract infection; XN: Xanthohumol. Figure created with BioRender.

3.3. Conclusion and Outlook

Overall, the findings of this dissertation demonstrate that fructose intake 1) alters intestinal barrier function and 2) alters TLR2-dependent activation of immune cells. In contrast, compounds such as IAA and xanthohumol derived from hop extracts attenuate pro-inflammatory cytokine responses by modulating downstream signaling pathways at doses found in foods and beverages. Importantly, these findings emphasize the importance of nutrition and support the concept that the food matrix can modulate the physiological effects of foods and beverages.

In conclusion, these results suggest that fructose-induced inflammatory alterations, including NO-dysregulation and enhanced TLR2-mediated signalling, may be modulated by hop-derived bioactive compounds such as IAA and xanthohumol. A key question for future research is how these fructose-induced immune effects, and their attenuation by bioactive dietary compounds, are modulated in the context of mixed diets and metabolically compromised cohorts. Addressing this question will be essential to elucidate dose-response relationships and to evaluate the long-term clinical relevance of these findings in larger human intervention studies.

4. Abstract

Die Zusammensetzung der Ernährung beeinflusst maßgeblich metabolische und entzündliche Prozesse und spielt eine zentrale Rolle bei der Entstehung der metabolisch bedingten steatotischen Lebererkrankung, sowie bei der Modulation der angeborenen Immunantwort. Insbesondere ein hoher Fruktosekonsum wird mit einer gestörten Darmbarrierefunktion und einer erhöhten Entzündungsanfälligkeit assoziiert. Gleichzeitig weisen aus Hopfen gewonnene bioaktive Verbindungen entzündungshemmende Eigenschaften auf, deren Wirkung beim Menschen in ernährungsrelevanten Dosen bislang unzureichend untersucht sind.

Ziel dieser Dissertation war es, die Mechanismen zu untersuchen, durch die Fruktose die intestinale Barrierefunktion und die Toll-like Rezeptor (TLR)-abhängige Immunantwort beeinflusst, sowie zu prüfen, ob Hopfenverbindungen wie Iso- α Säuren (IAA) und Xanthohumol entzündliche Signalkaskaden beim Menschen modulieren können. Hierzu wurden neben Untersuchungen im Mausmodell auch Humanstudien durchgeführt.

In einem Mausmodell führte eine fett- und fruktosereiche Ernährung zu einer gestörten Darmbarriere, einer Dysregulation der Stickstoffmonoxid-Homöostase und frühen Zeichen einer Leberentzündung. Ergänzende Humanstudien zeigten, dass die akute Fruktoseaufnahme die TLR2-abhängige Zytokinantwort von Monozyten verstärkt. Demgegenüber reduzierten IAA- und Xanthohumol-reiche Hopfenextrakte in Interventionsstudien die durch bakterielle Toxine induzierte Freisetzung proinflammatorischer Zytokine. Eine längerfristige Aufnahme von Xanthohumol in Form eines Xanthohumol-reichen Hopfenextrakts war zudem mit mildereren Erkältungssymptomen assoziiert.

Zusammenfassend zeigen die Ergebnisse dieser Arbeit, dass Fruktose die intestinale Barrierefunktion und die angeborene Immunantwort negativ beeinflusst, während ausgewählte Hopfenverbindungen entzündliche Signalwege abschwächen können.

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