Regulatory properties of lactic acid bacteria for improving immune homeostasis
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Summary

Even though consumption of lactic acid bacteria (LAB) has been recognized to offer multiple health benefits to the host, the precise mechanisms underlying the effects of individual strains are currently not well understood. Thus far, only a limited number of LAB strains with well-validated effects have been studied in dietary or medical interventions. Therefore, effective and high-throughput screening tools for identifying potential beneficial strains are urgently needed.

In chapter 1 we provide current insights in the possible mechanisms underlying the anti-infectious, gut barrier-strengthening, and immune-modulating effects of LABs. Specific strains may act via single or combinations of these mechanisms, which are largely dependent on their effector molecules. The effector molecules of LAB strains may be either present on bacterial cells or are secreted into bacterial culture supernatants. The effector molecules of LABs can serve as ligands for signalling receptors on the host cells, i.e. pattern recognition receptors (PRRs). The direct interactions between LAB-related ligands and PRRs have been considered as the molecular basis for LAB-induced physiological effects. Toll-like receptors (TLRs) were defined as one key family of PRRs that mediate the signalling response of LABs. The current evidence for the efficacy of LABs in modulating several diseases including pathogenic infections, intestinal inflammatory disorders, allergy, and cancer was reviewed. Despite the promising findings of specific LAB strains, contradictory results were observed. More molecular insights into the mechanisms that mediate the functional effects of LABs should be provided to better interpret the current findings and to facilitate more efficient applications of LABs.

In chapter 2 immune-regulatory properties of a range of LAB strains from various species were investigated. Different bacterial strains-elicited cytokine secretion profiles in THP1-differentiated human macrophages were first measured. We found that LAB-induced IL-6 and IL-10 production levels were species- and strain-specific. To provide more molecular insights in species- and strain-dependent stimulating effects of LAB in immune cell, we then examined the interactions of LAB with pattern recognition receptors (PRRs). Our results showed that PRR-activating effects of LAB were species- and strain-specific and that specific LAB strains signal via TLR predominantly TLR2 pathway. Both PRR- and TLR2-activating properties of LAB strains were stimulation dose-dependent. It was further demonstrated that all TLR2-signalling strains activated TLR2/TLR6 pathway but only one also had mild activating effects on TLR2/TLR1 pathway. Previous studies suggest that TLR2/TLR6 activation
contribute to inducing anti-inflammatory immune responses whereas TLR2/TLR1 activation is associated with triggering pro-inflammatory responses. Thus, those strains signal through solely TLR2/TLR6 may be potential candidate strains for ameliorating gut inflammatory disorders.

Not only LAB strains but also their secreted products were also suggested to have immune-active properties. Therefore, in chapter 3 secreted products of various LAB strains were investigated for their immunomodulatory effects. It was found that different bacterial culture supernatants induced species- and strain-specific cytokine (TNF-α or IL-10) production profiles in THP1 macrophages. Bacterial culture supernatants were further shown to signal primarily via TLR2 pathway in a species- and strain-dependent manner and their TLR2-activating capacities are correlated with their induced cytokine (TNF-α or IL-10) secretion levels. This suggests that interactions between bacterial secreted products and TLRs are the fundamental molecular basis that determines the immune effects of bacterial secreted products. Moreover, we found that secreted products of some strains induced different cytokine production and TLR activation profiles from bacterial strains. These findings indicate that apart from bacterial strains bacterial secreted products can also be applied in designing LAB-based functional food or pharmaceutical products for attaining particular health benefits.

Since TLR2 has been defined as a crucial PRR involved in maintaining gut epithelial barrier function, in chapter 4 the identified TLR2-activating LAB strains were investigated for their epithelial barrier-protective effects in T84 intestinal epithelial cells. To illustrate the underlying pathways involved in the barrier-protective effects of LAB strains, we applied two epithelial protein kinase C (PKC) pathway and mitogen-activated protein kinase (MAPK) pathway respectively. Only PKC-dependent disruptor induced trans-epithelial electrical resistance (TEER) decrease was effectively inhibited by pretreatment with TLR2-signalling LAB strains. These observations suggest that TLR2-activating bacterial strains conferred protection on intestinal epithelial barrier via influencing PKC signaling pathway.

Strengthening gut mucus barrier is reported as one key mechanism by which LABs contribute to the state of intestinal homeostasis. In order to investigate the potential effects of LABs on gut mucus function, we studied in chapter 5 effects of LAB strains on expression alterations of mucus-associated genes in goblet cells. Regulatory effects of LABs on goblet cell genes were species- and strain-specific and were also contingent on bacterial treatment
duration. Intriguingly, we also found that living bacteria are needed to be effective in impacting goblet cell gene transcription. Furthermore, the modulatory effects of several bacterial strains on mucus-related genes partially relied on their secreted bioactive factors. These findings indicate that the viability of LAB formulations should be carefully monitored to ensure their high efficiency in regulating goblet cell functions, and that bacterial secreted products can also be designed as functional foods for mucus barrier reinforcement. To explore whether LABs exert varied regulatory effects on goblet cell genes under different conditions in the gut, LABs-induced gene expression change in goblet cells were assessed in the homeostatic state and during exposure to TNF-α, IL-13 or the mucus synthesis inhibitor tunicamycin (Tm). Modulatory effects of LABs on mucus-related genes greatly relied on the applied stressor, implying that candidate strains for mucus enhancement should be determined based on the immune status of the target group.

Fibroblasts can interact with epithelial cells thereby playing a vital role in maintaining gut immune equilibrium. It is not fully clear whether fibroblasts impact mucus function or influence the regulatory effect of LABs on mucus. Therefore, in chapter 6 a goblet cell-fibroblast co-culture system was applied to explore the influence of fibroblasts on the transcription of mucus-related genes in goblet cells. We selected six LAB strains from different species with confirmed effects on mucus-associated genes and further studied the impacts of fibroblasts on the effects of these selected strains. Fibroblasts affected the expression of goblet cell genes and their effects were highly contingent on treatment duration and the bacterial species applied. The effects of fibroblasts were investigated under immune steady state and during stimulation with cytokine (TNF-α, IL-13) or the mucin synthesis inhibitor Tm. We observed that under different conditions fibroblasts conferred different effects on the expression of goblet cell genes. Our results confirmed the functional interactions of fibroblasts with goblet cells and their contributions to the gene expression in goblet cells. Thus, fibroblasts should be included in the in vitro cell models for evaluating the modulatory potentials of LABs on goblet cell functions.

The main findings of this thesis are discussed in chapter 7. We proved the species- and strain-dependency in the immune-stimulating, TLR-signalling, and mucus-regulatory effects of both bacterial strains and their released bioactive products. In chapter 2 and 3, our strategic approach of characterizing the immune-stimulating and TLR signalling effects of LAB strains or their
secreted products provides a technology platform for effective screening LAB strains that possess potential immune-regulating properties or secrete bioactive products with immune-active effects. Our approaches in chapter 4, 5, and 6 offer high-throughput selection systems for identifications of strain candidates with potential gut health-promoting effects. These findings will facilitate the development of novel nutraceutical products or functional foods.
**Nederlandse samenvatting**
Hoewel het bekend is dat de consumptie van melkzuurbacteriën vele gezondheidsvoordelen biedt, zijn de precieze mechanismen die ten grondslag liggen aan deze effecten van de verschillende stammen nog niet bekend. Tot op heden is er slechts een beperkt aantal melkzuurbacteriën bestudeerd in voedings- of medische interventies. Er zijn waarschijnlijk vele malen meer stammen met positieve effecten. Om deze te identificeren zijn er effectieve en high throughput screeningsplatformen nodig.

In **hoofdstuk 1** bieden we inzicht in de mogelijke mechanismen die ten grondslag liggen aan de anti-infectieuze effecten, de darm barrière versterkende effecten en immuun modulerende effecten van melkzuurbacteriën. Specifieke melkzuurbacteriën kunnen hun effect uitoefenen door middel van één of een combinatie van deze mechanismen. Dit is sterk afhankelijk van de expressie van hun effector moleculen. De effector moleculen van melkzuurbacteriën kunnen zowel aanwezig zijn op de bacterie cellen of worden uitgescheiden in het groeimedium. De effector moleculen van melkzuurbacteriën kunnen functioneren als liganden voor signaleringsreceptoren op de gastheercellen, zoals de zogenaamde **pattern recognition receptoren** (PRR). De directe interactie tussen melkzuurbacteriën gerelateerde liganden en PRRs worden beschouwd als de moleculaire basis voor melkzuurbacterie-geïnduceerde fysiologische effecten. Toll-like receptoren (TLoRen) vormen een belangrijke familie van PRRs die verantwoordelijk zijn voor de signaalrespons geïnduceerd door melkzuurbacteriën. De modulerende effecten van melkzuurbacteriën in verschillende ziektes, waaronder pathogene infecties, darm ontstekingsziekten, allergie en kanker worden in dit hoofdstuk samengevat. Ondanks de veelbelovende gezondheid bevorderende effecten van specifieke melkzuurbacteriën in bepaalde ziektes, zijn er ook veel tegenstrijdige resultaten gevonden. Daarom is er meer inzicht nodig in de moleculaire mechanismen die de functionele effecten van melkzuurbacteriën bewerkstelligen. Dit kan bijdragen aan het interpreteren van gevonden resultaten en aan meer efficiënte toepassing van melkzuurbacteriën mogelijk te maken.

In **hoofdstuk 2** zijn de immuun regulerende eigenschappen van geselecteerde melkzuurbacteriën stammen en soorten onderzocht. Verschillende melkzuurbacteriën, waaronder pathogene infecties, en melkzuurbacteriën gerelateerde liganden werden beschouwd als de moleculaire basis voor melkzuurbacterie-geïnduceerde fysiologische effecten. Toll-like receptoren (TLoRen) vormen een belangrijke familie van PRRs die verantwoordelijk zijn voor de signaalrespons geïnduceerd door melkzuurbacteriën. De modulerende effecten van melkzuurbacteriën in verschillende ziektes, waaronder pathogene infecties, darm ontstekingsziekten, allergie en kanker worden in dit hoofdstuk samengevat. Ondanks de veelbelovende gezondheid bevorderende effecten van specifieke melkzuurbacteriën in bepaalde ziektes, zijn er ook veel tegenstrijdige resultaten gevonden. Daarom is er meer inzicht nodig in de moleculaire mechanismen die de functionele effecten van melkzuurbacteriën bewerkstelligen. Dit kan bijdragen aan het interpreteren van gevonden resultaten en aan meer efficiënte toepassing van melkzuurbacteriën mogelijk te maken.
melkzuurbacterie soort en stam. Om meer moleculair inzicht te verkrijgen in de soort- en stamstimulerende effecten van melkzuurbacteriën in immuun cellen is de interactie van melkzuurbacteriën met PRRs onderzocht. Onze resultaten tonen aan dat PRR-activerende effecten van melkzuurbacteriën soort en stam specifiek waren en dat specifieke melkzuurbacteriën voornamelijk de TLR2 signaalroute activeren. Zowel PRR als TLR2-activerende eigenschappen van melkzuurbacteriën waren dosis afhankelijk.

Verder werd er aangetoond dat alle stammen die de TLR2-signaalroute activeerden dit deden via de TLR2/TLR6 route. Slechts één stam had een mild activerend effect op de TLR2/TLR1 signaalroute. Eerdere studies suggereren dat TLR2/TLR6 activatie bijdraagt aan anti-inflammatoire immuun responsen, terwijl TLR2/TLR1 activatie wordt geassocieerd met het induceren van pro-inflammatoire responsen. De melkzuurbacterie stammen die alleen signaleren via TLR2/TLR6 kunnen dus potentiële kandidaten zijn voor het verminderen van darm ontstekingsziekten.

Niet alleen melkzuurbacteriën, maar ook de door hun uitgescheiden producten kunnen immuun regulerende eigenschappen hebben. In hoofdstuk 3 hebben we daarom de immuun modulerende effecten van de uitgescheiden producten van melkzuurbacterie stammen bestudeerd. We vonden dat de supernatanten van bacteriekweken van verschillende soort- en stammen specifieke cytokine profiel (TNF-α of IL-10) induceerden in THP-1 macrofagen. Ook toonden we aan dat het bacteriekweek supernatant voornamelijk via de TLR2 signaalroute signaleert. Deze signalering is ook soort- en stam afhankelijk. De TLR2 activerende capaciteiten van de bacteriekweek supernatanten bleken gecorreleerd te zijn met hun geïnduceerde cytokine (TNF-α en IL-10) hoeveelheden. Dit suggerereert dat interacties tussen producten uitgescheiden door melkzuurbacteriën en TLRs de fundamentele moleculaire basis vormen voor de immuun effecten van deze bacteriekweek producten. Bovendien vonden we dat de uitgescheiden producten van enkele bacteriestammen verschillende cytokine expressie levels en TLR activatie profielen induceren. Deze bevindingen tonen aan dat de door melkzuurbacterie uitgescheiden producten, los van de bacterie zelf, ook zouden kunnen worden toegepast in het ontwerp van op melkzuurbacterie gebaseerde functionele voeding of farmaceutische producten met specifieke gezondheid bevorderende effecten.

Omdat TLR2 een cruciale PRR is die ook betrokken is bij het behouden van de barrière functie van het darm epitheel, hebben we in hoofdstuk 4 de epitheel barrière beschermend effecten van de TLR2-activerende
melkzuurbacteriën op T84 darm epitheel cellen bestudeerd. Om de onderliggende te bestuderen die betrokken zijn bij de barrière beschermende effecten van melkzuurbacteriën hebben we de cellen geincubeerd met twee barrière verstorende reagentia die aangrijpen op de epitheel protein kinase C (PKC) route en de mitogen-activated protein kinase (MAPK) route. Alleen het reagens dat de barrière verstoord via de PKC signaalroute en daardoor zorgt voor een afname in de trans-epitheel electrische resistentie (TEER) kon effectief geremd door een pre-incubatie van de cellen met TLR-2 activerende melkzuurbacteriën. Deze observaties suggereren dat TLR2-activerende bacterie stammen de darm epitheel barrière kunnen beschermen via interactie met de PKC-signalroute.

Versterking van de darm-barrière wordt beschouwd als één van de sleutelmechanismen waarmee melkzuurbacteriën bijdragen aan de darmhomeostase. Deze barrière wordt gevormd door mucus en epitheel. De effecten van melkzuurbacteriën op mucus aanmaak is nog nooit bestudeerd. Om de mogelijke effecten van melkzuurbacteriën op de darmslimfunctie te onderzoeken, hebben we in hoofdstuk 5 de effecten van melkzuurbacteriestammen op veranderingen in de expressie van mucus-geassocieerde genen in slijmbekercellen bestudeerd. De regulerende effecten van melkzuurbacteriën op genexpressie in slijmbekercellen waren soort- en stamspecifiek en waren ook afhankelijk van de incubatietijd met de bacteriën. Een interessante vinding was dat er levende bacteriën nodig zijn om de transcriptie van slijmbekercellen te beïnvloeden. Bovendien vonden we dat de modulerende effecten van verschillende bacteriestammen op slijm-gerelateerde genen gedeeltelijk berusten op de door bacterie uitgescheiden bioactieve factoren. Deze bevindingen geven aan dat de levensvatbaarheid van melkzuurbacteriën-formuleringen zorgvuldig moet worden gecontroleerd om hun hoge efficiëntie bij het reguleren van slijmbekercelfuncties te waarborgen, en dat producten uitgescheiden door de bacteriën ook kunnen worden gebruikt in het ontwerp als functioneel voedsel voor versterking van de mucusbarrière. Om te onderzoeken of melkzuurbacteriën verschillende regulerende effecten hebben op de genen van slijmbekercellen onder verschillende omstandigheden in de darm, werd de door melkzuurbacteriën geïnduceerde verandering in genexpressie in slijmbekercellen niet alleen vastgesteld onder homeostatische toestand, maar ook tijdens blootstelling aan TNF-α, IL-13 of de mucus-syntheseremmer tunicamycine (Tm). De modulerende effecten van melkzuurbacteriën op mucusafhankelijke genen waren sterk afhankelijk van de
toegepaste stressor, wat impliceert dat kandidaatstammen voor mucusversterking moeten worden bepaald op basis van de immuunstatus van de doelgroep.

Fibroblasten kunnen ook een interactie aangaan met epitheelcellen waardoor ze een vitale rol spelen bij het handhaven van het immuunhomeostase van de darm. Het is niet geheel duidelijk of fibroblasten de mucusfunctie beïnvloeden of dat ze de regulerende effecten van melkzuurbacteriën op mucus beïnvloeden. Daarom werd in hoofdstuk 6 een co-kweek systeem van slijmbekercellen en fibroblasten toegepast om de invloed van fibroblasten op de transcriptie van slijm-gerelateerde genen in slijmbekercellen te onderzoeken. We selecteerden zes verschillende melkzuurbacterie-stammen waarvan de effecten op met mucus geassocieerde genen bekend waren en bestudeerden de impact van fibroblasten op de effecten van deze geselecteerde stammen. Fibroblasten beïnvloedden de expressie van genen van slijmbekercellen. De effecten waren sterk afhankelijk van de duur van de behandeling met de bacteriën en van de toegepaste bacteriesoorten. De effecten van fibroblasten werden onderzocht onder zowel immuunhomeostase als tijdens stimulatie met cytokine (TNF-α, IL-13) of de mucinesynthese-inhibitor Tm. We hebben waargenomen dat fibroblasten onder verschillende omstandigheden verschillende effecten hebben op de expressie van genen van slijmbekercellen. Onze resultaten bevestigden de functionele interacties van fibroblasten met slijmbekercellen en hun bijdragen aan de genexpressie in slijmbekercellen. Derhalve moeten fibroblasten worden opgenomen in de in vitro celmodellen voor het evalueren van de modulerende effecten van melkzuurbacteriën op functies van slijmbekercellen.

De belangrijkste bevindingen van dit proefschrift worden besproken in hoofdstuk 7. We hebben de soort- en stamafhankelijkheid in de immuunstimulerende, TLR-signalerende en mucus-regulerende effecten van zowel bacteriestammen als hun vrijgekomen bioactieve producten aangetoond. De strategische aanpak in hoofdstuk 2 en 3 voor het karakteriseren van de immuun-stimulerende en TLR-signalerende effecten van melkzuurbacteriestammen en hun uitgescheiden producten bieden een platform dat gebruikt kan worden voor effectieve screening van melkzuurbacteriestammen met potentiële immuunregulerende eigenschappen of voor screening van bioactieve producten met immuunactieve bijwerkingen. De high-throughput systemen gebruikt in hoofdstuk 4, 5 en 6 bieden een selectieve manier voor identificatie van melkzuurbacteriestammen met
potentiële gezondheids bevorderende effecten op de darm. Deze bevindingen kunnen de ontwikkeling van nieuwe nutraceutische producten of functionele voedingsmiddelen vergemakkelijken.
Appendices

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任晟诚
Biography

Shengcheng (Chengcheng) Ren, the author of this thesis, was born on the 5th of August 1989 in Anhui, China. After completing her high school education (No.1 Senior High School of Xiaocheng) in 2006, she started her bachelor’s study at Qilu University of Technology from the same year. In 2010, she attained her bachelor’s degree with the major in Food Science and Engineering. Subsequently, she was awarded the Top Grade Award Scholarship from Jiangnan University and started the Successive Master’s-Doctoral Degree Program in the School of Food Science and Technology of Jiangnan University under the supervision of Prof. dr. W. Chen, Prof. dr. H. Zhang, and Dr. Q. Zhang. Since 2005, she moved to the Netherlands and started her PhD study in the Department of Pathology and Medical Biology, University Medical Center Groningen after obtaining the scholarship from China Scholarship Council (CSC). There she investigated the regulatory properties of lactic acid bacteria for improving immune homeostasis under the supervision of Prof. P. de Vos and Dr. M.M. Faas. This research project is also in collaboration with the Research Center of Food Biotechnology in Jiangnan University.
Publication list


