NFS Journal 14–15 (2019) 1–5

Contents lists available at ScienceDirect

NFS Journal

journal homepage: www.elsevier.com/locate/nfs

Gene expression changes as predictors of the immune-modulatory effects of probiotics: Towards a better understanding of strain-disease specific interactions

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ABSTRACT

Probiotic bacteria in clinical use grant a health benefit to humans when administered in adequate amount, frequency, and period. The majority of research into probiotics focuses on the usage of probiotics in the prevention and/or treatment of digestive diseases or other diseases related to an aberrant microbiota or inflamed mucosa. Hence, translational research often excludes the underlying multifaceted mechanisms of action of these supplements. This mini-review endeavours to summarize the mechanisms of action related to changes in gene expression, with a focus on studies published from 2015 to 2018. Alteration of gene expression has been described in the justification of the use of probiotics for certain diseases such as irritable bowel disease. The review centers on *in vivo* studies considering inflammation-related genes and pathways in gastrointestinal tissue and blood, and *in vitro* studies mainly from human intestinal epithelial cells but also immune cells. Probiotics are prospectively anti-inflammatory therapies in diseases with an impaired gut mucosa. Translational research will aim to target changes in genes expression that are strain- and disease-specific.

1. Introduction

The widely recognized definition of probiotic is: a collection of live microorganisms that, when administered in adequate amounts, confer a health benefit to the host. This description ignores other bacteria-secreted factors or inactivated bacteria that may have immunomodulatory properties [1–3]. The mechanisms of action for probiotics in the gastrointestinal tract include the up- and down-regulation of inflammation-related genes. The particular bacteria are identified in the gastrointestinal tract by pattern recognition receptors expressed by intestinal epithelial cells and local immune cells such as dendritic cells (DC). Included among the pattern recognition receptors are toll-like receptors (TLR) and C-type lectin receptors, which sit on the cell surface/endosome, and nucleotide-binding oligomerization domain (NOD)-like receptors of the cytosol. The gastrointestinal tract mucosa recognizes, through the aforementioned receptors, the microbe-associated molecular patterns expressed on probiotics and commensals [4]. The majority of available probiotics include the genera *Lactobacillus* or *Bifidobacterium* and have a direct or indirect immunomodulating communication. The direct mechanism involves for instance cell surface receptors (TLR, CLR) and occurs particularly in the small intestine where there is a thinner population of commensal bacteria [5,6]. While in the colon, probiotics act indirectly through changes of the local microbiota. Following the direct interaction, the probiotic induces changes in gene expression which, depending on the health of the mucosa, can trigger innate immunity. In human studies, evidence is not available for all digestive diseases although recent studies are promising for acute and chronic conditions such as irritable bowel disease, irritable bowel syndrome and diarrohea [7]. However, probiotics continue to be a potential support to gastrointestinal tract health as regulators of inflammation [4] and can be seen as both supplements and functional foods. The aim of this mini-review is to draw together some studies in probiotics concerning gene expression *in vitro* and *in vivo*, and considers only literature studies reporting mRNA data. It is a continuance of the well written systematic review by Plaza-Diaz et al. [8] with some differences: here are included only studies using single strains of probiotics to differentiate between the strain-specific effects. The studies included were conducted between 2015 and 2018 and regard *in vitro* human derived cells or clinical trials. The review considers examples of commonly used probiotics: lactic acid bacteria from the genera *Lactobacillus* and *Bifidobacterium*. *Lactobacillus*, bifidobacteria and some type of yeasts are a classification of probiotics based on the types of bacteria. However, classification of probiotics considering the host targets is not common. Hence the review wants to introduce the *genobiotics* definition. *Genobiotics* are defined as probiotics that has as main mechanism of action that brings to a clinical improvement the change in gene expression. For instance, if a probiotic that acts through NF-kB causes mucosal healing on the intestinal tissue then the probiotic

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https://doi.org/10.1016/j.nfs.2019.02.001
Received 14 January 2019; Received in revised form 5 February 2019; Accepted 6 February 2019
Available online 08 February 2019

is a NF-kB target bacteria.

2. Important pathways for inflammation-related genes

Changes in gene expression are among the first occurring changes on the human intestinal mucosa when exposed to a probiotic [5]. Alterations in gene expression are dynamic but *in vitro* and *in vivo* experiments have been commonly conducted to measure a cross-section of messenger ribonucleic acid (mRNA) and micro ribonucleic acid (miRNA) gene expression, for example using human expression microarrays or real-time polymerase chain reaction which targets single genes. Most probiotic strains of bacteria are gram positive bacteria and their *e.g.* peptidoglycans can interact with TLR inducing inflammatory cytokines such as tumour necrosis factor α, interleukin 1α, interleukin 1β, interleukin 6, interleukin 8, interleukin 10, TGFs [9,10]. Probiotics are most often reported to affect the following inflammation pathways in humans: TLR signalling pathway [11], NOD-like receptor signalling pathway [12], nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling pathway [3], myeloid differentiation primary response 88 (MyD88) pathway [13], janus kinases/signal transducer and activator of transcription proteins (JAK/STAT) signalling pathway [14] and p38 mitogen-activated protein kinases (p38 MAPK) pathway [3,15,16]. NF-kB signalling pathway is the most well-studied pathway. Hence, probiotics that act on this pathway could be defined as *genobiotics* subclass NF-kB acting bacteria. This pathway involves NF-κβ a transcription factor that positively regulates many genes that encode pro-inflammatory cytokines. It is suggested that more than one pathway could be targeting NF-κB modulation, indicating the complexity of the probiotics mechanisms of action [17]. In addition, probiotics may cause post-translational alterations and consequently gene expression changes involved in immune responses, such as induction of miRNA. Some miRNA such as miR-146a are known to regulate adaptor molecules in TLR/NF-κB signalling and are found to be differentially regulated in THP-1 immune cells between probiotics and pathogens [18]. Likewise mRNA, differential expression of gastrointestinal tract miRNA and downstream gene regulation is strain specific and time dependent [19].

3. Which genes/pathways are affected by which immune-beneficial strain? (Tables 1 and 2)

3.1. Bacteroides fragilis

The beneficial commensal *Bacteroides fragilis* passes molecules to the immune cells through secretion of outer membrane vesicles. *B. fragilis* outer membrane vesicles requires genes autophagy related 16 like 1 (ATG16L1) and NOD2 to induce regulatory responses in isolated T cells. The genes *ATG16L1* and *NOD2* are irritable bowel disease-associated genes necessary to overturn gastrointestinal tract inflammation during *B.fragilis* presence. In addition, *B.fragilis* contains a molecule named polysaccharide A (PSA). PSA is as well an anti-inflammatory molecule but unlike the bacteria does not require these specific genes for its anti-inflammatory action [11,20,21] .

3.2. Lactobacillus rhamnosus GG

Lactobacillus rhamnosus GG is reported to upregulate antagonists of NF-κB inflammatory pathway such as Vitamin D receptor signalling. Wu et al. postulates that *Lactobacillus rhamnosus GG* American type culture collection 53,103 increased the expression of the Vitamin D receptor target genes at a transcriptional stage in human intestinal epithelial cells HCT116. *Lactobacillus rhamnosus GG* and the bacteria conditioned media increased mRNA levels of cathelicidin in HCT116 cells. Vitamin D receptor target genes checked in this study were 25-hydroxyvitamin D3–24-hydroxylase gene (Cyp24) and antimicrobial peptides cathelicidin precursor. CYP24 gene is involved in Vit D catabolism [22].

Table 1
Study design of the human trials considered.

Human trial	Subjects	Sex	Protocol	Treatment	Duration	Sampling	Measurements and outcomes	Analyses
Chu et al. 2016 [21]	Crohn's disease, Healthy, *ex vivo*	Men, Woman	Control, blinded *ex vivo* stimulation	*Bacteroides fragilis*: Outer membrane vesicles (dose 5 μg), Polysaccharide A	18–22 h	Peripheral blood mononuclear cells	*ATG16L1 genotyping* and NOD2. *ATG16L1 is necessary for* Dendritic cells induction of CD4 + Foxp3 + IL-10+ Tregs when exposed to *Bacteroides fragilis* outer membranes vesicles.	Microarray (Immunochip)
Fong et al. 2016 [24]	Healthy *ex vivo*	N/A[b]	Control, blinded *ex vivo* stimulation	*Lactobacillus rhamnosus GG*: Bacteria (1×10^8 CFU[a]), Bacterial conditioned media (filtration 0.2 μm size)	24 h	Peripheral blood mononuclear cells	*TLR* 1, 2, 4, 5, 6, 7, 8, and 9	Real-time polymerase chain reaction
Solano-Aguilar et al. 2016 [23]	Elderly, *in vivo*	N/A[b]	Phase I open label study	*Lactobacillus rhamnosus GG*: Bacteria Oral administration of 1×10^{10} CFU[a] of bacteria per capsule twice daily	28 days	Whole blood cells	Transcriptomic profile: 95 differentially expressed genes; two genes, *FCER2* and *LY86*, down-regulated	**RNA-sequencing** Nominal significant difference (95 genes), stringent significance threshold (2 genes)

[a] CFU-Colony Forming Units.
[b] N/A- Not Available.

Table 2
Study design of the *in vitro* studies.

In vitro experiment	Cell type	Concentrations	Treatment	Duration	Controls	Measurements	Analysis
Wu et al. 2015 [22]	Human colon carcinoma HCT116	N/A[c]	*Lactobacillus rhamnosus GG:* Bacteria, Bacterial conditioned media	0–3 h	Unstimulated cells	cathelicidin, *CYP24,* antimicrobial peptides	Real-time polymerase chain reaction
Kalani et al. 2016 [25]	Human umbilical vein endothelial cell	$10^{9-}10^{10}$CFU[a]/mL	*Lactobacillus acidophilus:* Bacteria, Bacterial conditioned media, Bacteria water extract, Bacteria culture filtered, Bacteria unfiltered supernatant	24 h	Unstimulated cells	miRNA-21 miRNA-92a miRNA-155 miRNA-663	Real-time polymerase chain reaction
Xiong et al. 2018 [16]	Human epithelial colorectal adenocarcinoma Caco-2	0, 5, 10, 20 μg/mL	*Lactobacillus acidophilus:* Bacterial mucus-binding protein	*5 h*	N/A[c]	Interleukin 8, tumour necrosis factor α, TLR4, interleukin 1β, interleukin 10, NF-κB	Real-time polymerase chain reaction
Jiang et al. 2016 [3]	Human epithelial colorectal adenocarcinoma Caco-2	1×10^8 CFU[a]/mL	*Lactobacillus plantarum:* Bacteria	*0–24 h*	Negative control: Cell DMEM[b] media only	tumour necrosis factor α, interleukin 1β, interleukin 6, TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9	Real-time polymerase chain reaction
MacPherson et al. 2017 [26]	Human colorectal adenocarcinoma HT-29	Multiplicity of infection of 100:1 for the ratio bacteria to HT-29 cells	*Lactobacillus helveticus:* Bacteria, Surface-layer protein *Bifidobacterium longum:* Bacteria, *Bifidobacterium bifidum:* Bacteria	3, 6 h	N/A	Genome-wide human expression, TLR3 engagement	Microarray
Taibi et al. 2017 [19]	Human epithelial colorectal adenocarcinoma Caco-2	10:1 ratio bacteria to Caco-2 cells	*Bifidobacterium bifidum:* Bacteria	1, 4, 24 h	Vehicle alone	miRNA-148a, endothelial PAS domain protein 1	Real-time polymerase chain reaction
Widyarman et al. 2018 [27]	Immortalized human keratinocytes HaCaT	10^7 CFU[a]/mL	*Lactobacillus reuteri:* Bacteria	3, 6 h	N/A	interleukin 8, human-beta-defensin 2	Real-time polymerase chain reaction

[a] CFU-Colony Forming Units.
[b] DMEM-Dulbecco's Modified Eagle Medium.
[c] N/A- Not Available.

Consistent with these data, a clinical trial investigated the transcriptome of whole blood cells during 28 days *Lactobacillus rhamnosus GG* American type culture collection 53,103 intake. When comparing their data with other published studies on *Lactobacillus rhamnosus GG*, *In vivo* transcriptome changes in whole blood cells showed to imitate the response induced by proteasome blocker drugs. Proteasome blockers confer anti-inflammatory properties by lowering the activation of pro-inflammatory NF-κβ [23]. An additional study using blood explored the effects of *Lactobacillus rhamnosus GG* soluble factors *versus* conditioned media suggesting similarity on immunomodulatory effects by *e.g.* involving expression of TLR which increased in all cell types. In addition, initiation markers on monocytes and macrophages and production of interleukin 10, interleukin 12 and tumour necrosis factor α in macrophages declined [24].

3.3. Lactobacillus acidophilus

Probiotics are considered anti-inflammatory in atherosclerosis processes and might have a regulatory function of endothelial cell dysfunctions led by miRNA. An Iranian study investigated *Lactobacillus acidophilus* American type culture collection 4356 outcome on gene expression of miRNA-92a, miRNA-21, miRNA-155, and miRNA-663. Human umbilical vein endothelial cell (HUVEC) stimulated with bacterial lipopolysaccharide (LPS) showed an increase in miRNA-21 expression, gene with a role in apoptosis. However, conditioned media of *L-acidophilus*, among other, decreased the inflammatory miRNA-155 [25]. Considering intestinal epithelial cells Caco-2 instead *L. acidophilus* was proposed to exert its anti-inflammatory mechanism *via* TLR2 and TLR4 receptors gene expression [32]. Furthermore, the isolated mucus-binding protein of *L. acidophilus* triggered immune responses and intestinal protection by reducing the release of inflammatory cytokines interleukin 8 and increased the expression of interleukin 10. TLR4 and MAPK signalling pathways were necessary for immunomodulation [16].

3.4. Lactobacillus plantarum

Jiang et al. proposes *L.plantarum* NDC 75017 to be immunomodulatory because of an induction associated to NF-κB and p38 MAPK pathways. In Caco-2 cells stimulated with L. *plantarum* NDC 75017, the cytokines interleukin 1β, tumour necrosis factor α, interleukin 6 were rapidly produced and a consequence of a transient activation of a number of TLR expressed as gene fold change. Phosphorylation of NF-κB p65 and p38 MAPK were detected in Caco-2 cells confirmed after NF-κB and p38 MAPK inhibitors significantly inhibited cytokine expression [3].

3.5. Bifidobacterium bifidum and Bifidobacterium longum

A genome-wide transcriptional analysis study considered the transcriptional response of HT-29 cells stimulated with *Lactobacillus helveticus* R0052 (Lh-R0052), *Bifidobacterium longum* subsp. *infantis* R0033 (Bl- R0033) and *Bifidobacterium bifidum* R0071 (Bb-R0071). The study considered also probiotic combination (PC) and a purified Lh-R0052-SLP in the presence of the TLR3 synthetic analog polyriboinosinic:polyribocytidylic acid (poly(I:C)). The probiotics gave a diverse gene phenotype when faced with the TLR3 analog. From each of the probiotics, Bb-R0071 measured a higher number of genes of tumour necrosis factor signalling pathway expressed as number of up- down-regulated genes, Lh-R0052 of MAPK signalling pathway and NF-κB pathway, while Bl- R0033 affected compound and immune related pathways [27]. In the same year Taibi et al. aimed to determine if bifidobacteria-host crosstalk *in vitro* involves miRNA. Exposure to B. bifidum MIMBb75 but not to B. bifidum NCC390 or B. longum NCC2705, increased the expression of miRNA-148a after 1 and 4 h but not 24 h. The increase in miRNA-148a was accompanied by a decrease in endothelial PAS domain-containing protein 1 (*EPAS1*) expression. And, silencing of miRNA-148a reversed B. bifidum MIMBb75 dependent downregulation of EPAS1 associated inflammatory response [19].

3.6. Lactobacillus reuteri

In a focused study glycerol-supplemented *Lactobacillus reuteri* American type culture collection 55,730 was shown to reduce the expression of interleukin 8 and human-beta-defensin 2 (*hBD-2*) caused by *Streptococcus mutans* [27].

4. Digestive diseases and inflammation

The study of mechanisms of action of probiotics with immunomodulatory potential are important because probiotic bacteria can be considered for adjuvant anti-inflammatory management in those conditions where the immune system is particularly exacerbated (*e.g.* irritable bowel disease). Identifying gene-strain specific relationships for probiotics could help to target mRNA gene specific changes in inflammatory diseases. For instance, interleukin 1β response is particularly amplified in necrotising enterocolitis. Thus, identification of probiotic strains and secreted factors that can affect interleukin 1β associated pathways can be a potential target for necrotising enterocolitis interventions [28]. In addition to mRNA, miRNA includes possible gene regulators of importance for instance in irritable bowel disease. Following irritable bowel disease modulation, miRNA expression is hypothesised to be affected by strain-specific bacteria and hence impact the gastrointestinal tract immune response. For instance, endogenous miRNA-155 and miRNA-146a modulate responses by undergoing a functional transfer between immune cells and regulate inflammatory communication channels [29]. In clinical studies of irritable bowel disease involving drugs, mucosal gene expression analyses are being performed in parallel to the classical immunohistochemistry analysis from the same patients' biopsies. While immunohistochemistry data relate directly to measurable clinical symptoms, such as mucosal healing, gene expression profiles are unique based on inflammation status and patient. When remission from the disease targeted with the drug is achieved only for a number of patients, it is useful to check the expression of immune-related genes in responders to see which specific genes were mainly involved in mucosa healing [30]. A similar concept has been adopted for *response based mind-body group intervention,* a type of non-pharmacological therapy for irritable bowel disease and irritable bowel syndrome. The study showed that in irritable bowel disease this non-pharmacological therapy, involving clinical symptoms reduction such as relaxation, reduced expression of genes that were linked to, among others, inflammation in blood samples [31]. These types of studies are important because they are unravelling the gene changes for irritable bowel disease that give disease remission and disease cure. On one hand is important to identify these disease specific gene changes and on the other targeting these changes with drugs and/or probiotics. So far no drug has been successful in complete remission of irritable bowel disease. Knowing that probiotics affect gene expression, if the same concept can be applied to studies with probiotics aiming to target specific gene expression changes, then we would have adjuvant therapies for cure and maintenance. Therefore, targeted-probiotics that here we introduce as *genobiotics* are a proposed way to look at probiotics classified based on genes they target in humans diseases.

5. Concluding remarks

Probiotics can be integrated as a new potential immunoregulatory supplement adjuvant to anti-inflammatory therapies excluding the flare time point of *e.g.* irritable bowel disease. For this purpose, transitional research investigating the mechanism of action is needed. Gene expression data are a good screenshot of the first moment the probiotic

gets in contact with the human cells (*in vitro*). Gene expression can be related to distinguishable changes of clinically relevant outcomes (*in vivo)* such as mucosa healing. For this, strain-pathway/gene specificity is crucial in understanding translational changes of the complex human-microbiota interaction. Hence, is *genobiotics* a new term to classify probiotics based on host changes in gene expression?

Funding and acknowledgments

This review was funded by the Operational Scholarship (Driftstöd) at Örebro University, Orebro, Sweden. The author thanks Dr. Stephen T.A Rush for proof-reading the paper and the editor/reviewers for the critical revision.

Conflict of interest

No potential conflict of interest was reported by the author.

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