Aloe barbadensis Mill. as a therapeutic option for irritable bowel syndrome

– properties, bioactivity and mode of action

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Gothenburg 2022

Cover illustration: *Gut Relief* by Jimmy Ahluwalia

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ISBN 978-91-8009-987-5 (PRINT)
ISBN 978-91-8009-988-2 (PDF)
http://hdl.handle.net/2077/72575

Printed in Borås, Sweden 2022
Printed by Stema Specialtryck AB

To my Akira and Aveer

ੴ A special thank you to my guiding stars Nanu and Dad-in-law

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ABSTRACT

Irritable bowel syndrome (IBS) is a chronic and prevalent functional gastrointestinal disorder, with an incompletely understood pathophysiology. Because of the disease complexity and heterogeneity, the currently available treatment options for IBS are limited. These limitations have led to the popularity of alternative therapeutic strategies, such as the use of *Aloe barbadensis* Mill. (Aloe), despite the paucity of controlled clinical studies supporting efficacy of these treatment options. This thesis therefore aimed to determine the importance of intestinal microenvironment, as well as the therapeutic effects and potential mode of action of an Aloe gel derived extract in patients with IBS.

An integrated faecal microbiota and metabolite profile, as a joint representative of the intestinal microenvironment, distinguished IBS patients from healthy subjects, and further established the role of an altered intestinal microenvironment in the pathogenesis of IBS. The overall safety of Aloe treatment in patients with IBS was confirmed and supported the beneficial treatment effect of Aloe gel extract in subsets of IBS patients, which may depend on gut microbiota composition and function. Further, a potential mode of action for the therapeutic effect of Aloe gel extract, including dampening of immune cell activity and modulating intestinal microenvironment, was proposed. Finally, with the help of metabolomics, we expanded the knowledge of the complex and synergistic bioactive composition of Aloe gel.

In conclusion, this thesis strengthens the role of an altered intestinal microenvironment in the pathogenesis of IBS. Further, it supports the role of an Aloe gel derived extract as a therapeutic option for the symptom management of IBS.

Keywords: *Aloe barbadensis* Mill., Aloe, irritable bowel syndrome, metabolites, microbiota, prebiotic, immunosuppressive

ISBN 978-91-8009-987-5 (PRINT)
ISBN 978-91-8009-988-2 (PDF)

SAMMANFATTNING PÅ SVENSKA

Irritable bowel syndrome (IBS), är en kronisk funktionell mag-tarmsjukdom med oklar patofysiologi. På grund av sjukdomens komplexitet och heterogenitet är behandlingsalternativen vid IBS begränsade. Detta har lett till ett intresse för bland annat *Aloe barbadensis* Mill. (Aloe), trots bristen på kontrollerade kliniska studier som stöder dess effekter. Denna avhandling syftade därför till att klargöra betydelsen av tarmens lokala mikromiljö, samt behandlingseffekter och verkningsmekanismer av ett extrakt från Aloe, hos patienter med IBS.

En integrerad profil bestående av fekal mikrobiota och metaboliter, vilka tillsammans representerar tarmens lokala mikromiljö, särskilde IBS patienter från friska försökspersoner. Vidare bekräftades att behandling av IBS patienter med Aloeextrakt är säker. En grupp av patienter med IBS rapporterade en gynnsam behandlingseffekt av Aloeextraktet, vilket kunde kopplas till mikrobiotans sammansättning och funktion i tarmen. Vidare presenterades verkningsmekanismer för behandlingseffekten av Aloeextrakt, vilka inkluderade dämpning av immuncellsaktivitet och modulering av tarmens lokala mikromiljö. Slutligen, med hjälp av metabolomik, förbättrades kunskapen om Aloes komplexa och synergistiska bioaktiva komposition.

Sammanfattningsvis styrker denna avhandling betydelsen av tarmens lokala mikromiljö för patogenesen vid IBS. Vidare stöder den användning av Aloeextrakt som behandling för att lindra symptom hos patienter med IBS.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Ahluwalia B**†, Iribarren C†, Magnusson MK, Sundin J, Clevers E, Savolainen O, Ross AB, Törnblom H, Simrén M, Öhman L. *A distinct faecal microbiota and metabolite profile linked to bowel habits in patients with irritable bowel syndrome.*
Cells. 2021; 10(6): 1459.

II. **Ahluwalia B**, Magnusson MK, Böhn L, Störsrud S, Larsson F, Savolainen O, Ross A, Simrén M, Öhman L. *Randomized clinical trial: Effects of Aloe barbadensis Mill. extract on symptoms, fecal microbiota and fecal metabolite profiles in patients with irritable bowel syndrome.*
Neurogastroenterol Motil. 2020; 32(8): e13860.

III. **Ahluwalia B**, Magnusson MK, Böhn L, Störsrud S, Larsson F, Öhman L and Simrén M. *Aloe barbadensis Mill. extract improves symptoms in IBS patients with diarrhoea: post hoc analysis of two randomized double-blind controlled studies.*
Therap Adv Gastroenterol. 2021; 8(14): 17562848211048133.

IV. **Ahluwalia B**, Magnusson MK, Isaksson S, Larsson F, Öhman L. *Effects of Aloe barbadensis Mill. extract (AVH200®) on human blood T cell activity in vitro.*
J Ethnopharmacol. 2016; 179: 301-9.

V. **Ahluwalia B**, Magnusson MK, Larsson F, Savolainen O, Ross AB, Öhman L. *Differences in metabolite composition of Aloe barbadensis Mill. extracts lead to differential effects on human blood T cell activity in vitro.*
Molecules. 2022; 27(19): 6643.

† Shared first co-authorship

CONTENT

ABBREVIATIONS

BSF	Bristol stool form
FGID	Functional gastrointestinal disorder
FODMAP	Fermentable oligo-, di-, and monosaccharides and polyols
GI	Gastrointestinal
GC-MS/MS	Gas chromatography – tandem mass spectrometry
HAD	Hospital Anxiety and Depression
HPLC-UV	High-performance liquid chromatography- ultra-violet
IASC	The International Aloe Science Council
IBS	Irritable bowel syndrome
IBS-C	IBS with predominant constipation
IBS-D	IBS with predominant diarrhoea
IBS-M	IBS with mixed bowel habits
IBS-U	IBS unclassified or unsubtyped
IBS-SSS	IBS Severity Scoring System
IEC	Intestinal epithelial cell
IL	Interleukin
LC-MS	Liquid chromatography-Mass spectrometry
NMR	Nuclear magnetic resonance
OPLS-DA	Orthogonal partial least square-discriminant analysis
PBMC	Peripheral blood mononuclear cells

PCA	Principal component analysis
ppm	Parts per million
SCFAs	Short chain fatty acids
TLR(s)	Toll-like receptor(s)
Th	T helper cells
Tregs	Regulatory T cells
16S rRNA	16S ribosomal RNA

PREFACTORY

Irritable bowel syndrome (IBS) is a chronic and prevalent functional gastrointestinal (GI) disorder, with an incompletely understood pathophysiology. As a consequence of the disease complexity and heterogeneity, the currently available treatment options for IBS are limited. These limitations have led to the popularity of alternative treatment strategies for the management of IBS, including the use of medicinal foods and herbal products like *Aloe barbadensis* Mill. (Aloe), despite the paucity of controlled clinical studies supporting efficacy of these treatment options.

The overall aim of this thesis was to explore our hypothesis that the intestinal microenvironment is important in the pathophysiology of IBS and that *Aloe barbadensis* Mill. (Aloe) gel derived extract has beneficial therapeutic effects in patients with IBS (Figure 1). More specifically:

Paper I assessed the importance of alterations in the intestinal microenvironment, represented by the combined faecal microbiota and metabolite profile, in patients with IBS compared to healthy subjects.

Paper II and **Paper III** determined the effects of Aloe gel extract intervention in IBS patients with regards to GI symptoms and modulation of the intestinal microenvironment.

Paper IV assessed the effects of Aloe gel extract on immune cell activity *in vitro* as a potential underlying mechanism of action.

Paper V compared the effect of various commercially available Aloe gel extracts on immune cell activity *in vitro* and correlated this effect to their distinct metabolite composition.

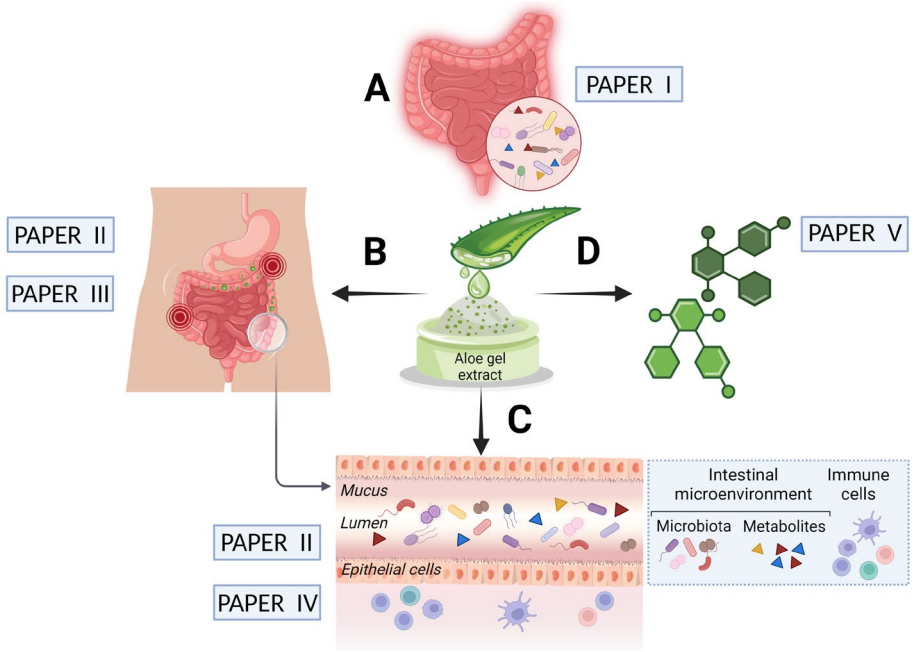

Figure 1. Graphical summary of the overall aim of this doctoral thesis. *The general aim was to determine the importance of the intestinal microenvironment in IBS (A) and to assess the therapeutic effect of Aloe barbadensis Mill. (Aloe) extract intervention in patients with IBS (B). Moreover, we evaluated whether potential effects of Aloe gel derived extract may be mediated by altering immune cell activity and modulating the intestinal microenvironment (C), as well as aimed at characterising the bioactive composition of Aloe extract responsible for its effect on immune cell activity in vitro, as a potential underlying mechanism for its therapeutic effect in patients with IBS (D). Created with BioRender.com*

THE GASTROINTESTINAL TRACT

The human gastrointestinal (GI) tract is a system of multiple organs including the mouth, oesophagus, stomach, small intestine, large intestine and rectum, which form a continuous channel through the body.[1] It also homes a complex community of commensal microbes, collectively referred to as the microbiota.[2] Being the largest interface between the body and the external environment, the GI tract is in constant contact with an immense load of antigens including microbes, dietary antigens as well as self-antigens.[3] Thus, it is not surprising that it is also closely associated with a complex innate and adaptive immune system.[3-6] While the primary function of the GI tract is digestion of food and uptake of nutrients, it also has the daunting task of shielding the body from potentially harmful pathogens, while keeping tolerance to food, commensal microbes and self-antigens, or in other words maintaining immune homeostasis.[4,7] This dual role of the GI tract is facilitated by its unique architecture and composition of specialised immune cells.[4]

The continuous layer of epithelial cells making up the inner mucosal layer, which differs in structure and function depending on their location in the GI tract, forms the basic architecture of the gut. The vast surface area of the intestinal epithelium monolayer, which results from the multiple folding villi, lined with intestinal epithelial cells (IECs) that have microvilli, help to optimise the absorption of nutrients.[1,4] Along with the epithelium, the functional anatomy of the GI tract includes a sub-mucosal layer composed of nerves, lymphatics, and connective tissues.[1] Although beyond the scope of this thesis, the proper functioning of the gut also involves the complex communication between the nerve network of the gut known as the enteric nervous system and the central nervous system. This bidirectional interaction between the gut and brain is now widely known as the “gut-brain axis” and is essential for GI functions including digestion, motility, and mucosal immune response.[1,8] Further, recent evidence also suggests the importance of the gut microbiota in influencing gut-brain interactions.[8]

The physical and immunological integrity of the intestinal barrier along with its interaction with the gut microenvironment plays an important role for the proper functioning of the GI tract and supporting health in general. Impairment in the intestinal barrier, mucosal immune system

defects, and altered gut microenvironment are aspects that may be involved in the pathogenesis of GI diseases including irritable bowel syndrome (IBS), the focus of this thesis, and will be discussed further.

THE INTESTINAL EPITHELIAL BARRIER

The GI tract is a complex organ that harbours an intricate innate and adaptive mucosal immune system, while also being colonised with trillions of beneficial commensal bacteria, which play an important role in the development and function of the mucosal immune system.[9] This coexistence requires barrier and regulatory mechanisms, which are mediated by the intestinal epithelium, functioning as a crucial moderator of intestinal homeostasis.[9]

The intestinal epithelium composed of a single layer of IECs, makes up the body's largest mucosal surface.[9] The IECs comprising of five distinct cell types namely, enterocytes, endocrine cells, microfold cells, goblet cells and Paneth cells, form the outmost layer of the intestine. This layer of cells together with the mucus layer form a physical and biochemical barrier to microorganisms, both pathogenic and commensal, as well as defence against antigens, allergens, and toxins.[10] Antimicrobial peptides and mucins secreted by the specialised secretory IECs (Paneth cells and goblet cells) further limit the bacteria that reach the epithelial surface and encounter the underlying mucosa.[9,11]

Mucins, the main building blocks of the mucus layer, vary considerably in their organisation throughout the GI tract. The mucus layer of the intestine is built around the gel-forming mucin MUC2, which is produced by the goblet cells.[12,13] Although MUC2 is the main structural component of the mucus layer in both the small and the large intestine, the properties at these two locations are very different. The small intestine has only one layer of non-attached mucus, rich in antibacterial peptides and proteins from the Paneth cells. The large intestine on the other hand, has a two-layered mucus system, with a less dense and unattached outer layer, as well as a dense, more organised inner layer, being impermeable to the luminal bacteria. Altogether, the mucus layer physically separates the epithelial cell surface from bacteria and adds to the protective function of the intestinal barrier.[12,13]

While forming a segregation between the gut lumen and its contents (external environment) from the lamina propria (internal

environment), the intestinal epithelium also allows permeability of water, electrolytes, and nutrients. This dynamic permeability is a result of a complex network of tight junction proteins consisting of claudins, occludins, zonula occludens and junction adhesion molecules, which while enabling permeability to water and nutrients, help to create a seal between neighbouring IECs and prevent the entry of pathogens.[14,15]

To accomplish intestinal homeostasis, the intestinal epithelium also acts as a regulator of innate and adaptive immune responses in the GI tract, likely through its interaction with the underlying immune cells and the gut microbiota.[16,17] The IECs can sense microbial stimuli with the help of various pattern-recognition receptors, which along with reinforcing the barrier function also help in coordinating signals derived from commensal and pathogenic bacteria and hence induce an appropriate tolerogenic or pro-inflammatory response.[18] Furthermore, specialised cells called the intraepithelial lymphocytes, are present between adjacent epithelial cells. Due to the proximity of the intraepithelial lymphocytes to gut antigens they protect the mucosal barrier against pathogens and have also been implicated in maintaining gut homeostasis.[19]

THE IMMUNE SYSTEM OF THE GUT

Harbouring around 70% of the body's lymphocyte population, the GI tract is considered to be the largest immunological organ in the body.[6] Along with the intestinal epithelial barrier, the immune cells residing in the underlying loosely packed connective tissue called the lamina propria and the gut-associated lymphoid tissue, composed of Peyer's patches, isolated lymphoid follicles and mesenteric lymph nodes, altogether make up the mucosal immune system of the gut.[4,5] The complexity of the gut immune system although beyond the scope of this thesis, is described in brief below and can be read in detail in the review titled "Mucosal immune system of the gastrointestinal tract: maintaining balance between the good and the bad" by Ahluwalia *et al.* (2017).[7]

The intestinal epithelium along with the lamina propria is the largest site for the freely dispersed T cells, the majority being antigen experienced lymphocytes. Together they share the important role of being the effector site of the intestinal immune response, preventing entry of pathogenic organisms and their destruction in case of an

invasion.[7,20] In addition to lymphocytes, the lamina propria also harbours a rich network of innate immune cells including dendritic cells, macrophages, mast cells and eosinophils among others.

Peyer's patches contain B cells, T cells, macrophages and dendritic cells organised into lymphoid follicles. Along with the mesenteric lymph nodes, which harbour a large number of lymphocytes, antigen presenting cells and macrophages, they make up the gut-associated lymphoid tissue, the main inductive site where the antigens from the mucosal surface activate an adaptive immune response.[21]

The Peyer's patches, more frequent in the ileum, enable sampling and transport of luminal antigens and intact bacteria to dendritic cells, the main antigen presenting cells in the gut-associated lymphoid tissue. This important step in initiation of the immune response is mediated by microfold cells found in the follicle-associated epithelium, lining the Peyer's patches and separating them from the lumen.[22] Along with the microfold cells as the key site for antigen uptake, there are several other potential routes for antigen entry and presentation, including direct sampling by the dendritic cells, which can extend their dendrites between the epithelial cells and reach the lumen, as well as passage of small molecules via the goblet cells.[23,24] Recognition of microbial invaders by innate immune cells including neutrophils, eosinophils, macrophages, mast cells and dendritic cells leads to the secretion of inflammatory cytokines, chemokines, antimicrobial peptides, and activation of dendritic cells, hence forming the first line of defence in case of invasion.[4,25] Dendritic cells following uptake of antigens, become activated into potent antigen presenting cells. These antigen presenting cells then activate lymphocytes locally in the Peyer's patches or in other locations in the gut-associated lymphoid tissues. The activation and differentiation of T cells at the inductive sites are mediated by antigen specific signals and the local cytokine milieu into T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17) cells, and regulatory T cells (Tregs). Further, activation of B cells and differentiation into plasma cells also takes place in the gut associated lymphoid tissue, after interaction with antigen presenting cells and T cells.[4,26,27]

The activated effector T lymphocytes and plasma cells then leave the inductive sites and migrate towards the effector sites, where they exert protective immune responses or tolerogenic immune response, depending on the type of antigen and stimuli received from the

intestinal environment. Along with the properties of the antigen, specialised subsets of dendritic cells traveling within and between the inductive and effector sites of the gut-associated lymphoid tissue, also play an important role in the decision between inducing inflammation or tolerance.[28] While the mucosal immune system of the gut can generate a protective immune response, it has a predisposition to a tolerogenic response, which helps it to prevent inappropriate inflammation against food antigens or commensal bacteria. Dendritic cells influenced by immunoregulatory signals from their interaction with other immune cells including epithelial cells, develop tolerogenic properties.[28] These conditioned dendritic cells produce IL-10 which promotes differentiation of naive T cell into Tregs, as well as maturation of B cells into IgA producing plasma cells, which are involved in tolerogenic responses and maintenance of gut homeostasis.[29]

The mucosal immune system of the gut is complex and multifaceted, and as our understanding of the gut immune system advances, it is becoming more evident that, besides the multiple components discussed above, their interaction with the intestinal microenvironment including the gut microbiota play an important role in the development and modulation of the gut immune responses.[30-32]

THE INTESTINAL MICROENVIRONMENT

The human GI tract is a multifaceted environment that is home to an enormous and complex community of commensal microbes, including bacteria, archaea, eukaryote, and viruses, which are collectively referred to as the gut microbiota.[31,32] The dynamic crosstalk between the host and gut microbiota is essential for maintaining normal gut function as well as host health in general. This complex interaction involving the microbes, the host epithelium and the host immune system, is part of an intricate ecosystem which gives rise to diverse metabolites and small molecules produced by the microorganisms, dietary metabolites as well as molecules produced by the host, which all together make up the "intestinal microenvironment".[2,31,32] Microbiota, and in particular metabolites, are detectable in a range of biological samples. Despite the known compositional differences between faecal and mucosal microenvironment, in this thesis faecal samples, due to the ease of collection, were analysed for microbiota and metabolite composition as a proxy for the intestinal microenvironment (**Paper I and II**).

Gut Microbiota

The GI tract hosts more than 100 trillion microorganisms, estimated to encompass more cells compared to human cells and hence makes up an important element of the intestinal microenvironment.[2,33,34] The gut microbiota, which are permanent residents in humans, have co-evolved with their human host to form a mutually beneficial relationship. The gut microbiota contributes to many physiological functions, such as aiding in digestion, production of nutrients, and most importantly development and regulation of the host mucosal immune system, along with protection against pathogens. In return, the host offers nourishment and habitat to the gut microorganisms.[2,33,34] The importance of the gut microbiota in development of immunity and protection has been established through studies using germfree and gnotobiotic animal models, which showed impaired development of the gut-associated lymphoid tissue, compared to conventional animal models.[30,35,36]

The development of the gut microbiota begins at birth and continues to develop in composition, diversity, and functional ability until around 2-3 years of age. At this age the infant microbiota is considered to resemble the adult microbiota, or the core native microbiota, which despite being relatively stable, can be altered by perturbations in life events such as infections and use of antibiotics.[34,37] While several factors contribute to the initial composition and diversity of the gut microbiota, including delivery type, as well as feeding and weaning period, the microbiota continues to diversify with lifestyle, cultural habits, environmental factors and diet which exerts a large effect.[38] The gut microbiota also tends to differ between different geographical regions and developmental stages, with ageing further affecting composition of gut microbiota.[39] However, the variability of microbiota is known to be higher between individuals than the fluctuation seen within the same individual over time.[40,41]

The concentration of the gut microbiota increases along the GI tract with the colon being the most densely populated.[40] The composition of the gut microbiota also varies depending on its location in the GI tract. Bacteria belonging to phyla Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria and Verrucomicrobia dominate the adult gut microbiota. In more detail, the healthy adult microbiota is dominated by Firmicutes (*Lactobacillus*, *Bacillus*, *Clostridium*, *Enterococcus*, and *Ruminococcus*; with *Clostridium* genera representing 95% of the

Firmicutes phyla), Bacteroidetes (*Bacteroidaceae*, *Prevotellaceae,* and *Rikenellaceae*), and Actinobacteria (*Bifidobacteriaceae* and *Coriobacteriaceae*).[40,42,43] Several microorganisms belonging to these families at the genus level including *Clostridium, Bifidobacterium, Bacteroides* and *Prevotella,* and *Akkermansia* have further been associated with health.[42] Despite evidence of the importance of gut microbiota in human health, researchers are still discussing the vital question – 'What shapes a healthy gut microbiota?'. Our current understanding indicates that there is no universally healthy microbiota or 'the perfect' microbiome; in fact, the optimal healthy gut microbiota composition differs between individuals. Additionally, along with which microbes are present in the gut, researchers are now focusing on their function to understand how the microbes are communicating with each other and interacting with the host. In this regard, changes in the abundance or functional roles of these microorganisms, have been associated with development of GI diseases and disorders and hence is of interest for this thesis.

Microbial metabolites

Another beneficial contribution of the gut microbiota to host physiology and function, is the metabolites or small molecules produced by gut microbes through the anaerobic fermentation of undigested dietary components.[44] These microbial metabolites play an important role in a myriad of host health functions including energy and signalling pathways, metabolic reactions and most importantly host immune system development and function.[32] Gut microbiota uses microbial metabolites as key mediators for communicating, educating, and stimulating the host, hence these metabolites are often considered as the functional output or a snapshot of the complex host-microbial interactions.[32,44]

The production of microbial metabolites is driven by a combination of factors, including dietary substrate availability, luminal microenvironment, including microbiota composition, as well as variability in host physiology such as intestinal transit time.[44] Short chain fatty acids (SCFAs) such as acetate, propionate and butyrate, produced by the gut microbial fermentation of dietary fibres are cardinal examples of metabolites related to health benefits and are amongst the most studied metabolites. Along with SCFAs, the gut

microbiota produces many other types of metabolites, such as secondary bile acids; amino acids such as tryptophan, glutamate, glycine and ornithine; indole derivates and polyamines, such as putrescine and spermidine, that also have essential signalling functions and play an important role in maintaining host-microbiota homeostasis.[44,45]

Regarding gut bacteria, *Faecalibacterium* and C*lostridium* spp. belonging to the Clostridial clusters, *Bifidobacterium* spp. from Actinobacteria class, *Akkermansia muciniphila* belonging to phylum Verrucomicrobia, and *Lactobacillus* belonging to phylum Firmicutes are some bacteria which are known to play an important role in SCFAs metabolism, as well as production of many other beneficial metabolites.[46]

The complex crosstalk between the microbiota composition, their metabolites and other luminal components with the host is necessary not only for immune homeostasis, but also influences the susceptibility of the host to many diseases and disorders. Hence, alterations in metabolite production in the gut, caused by disturbances in host-microbiota interaction, have been implicated in many diseases including IBS and are thus of importance for this thesis.

IRRITABLE BOWEL SYNDROME

Irritable bowel syndrome (IBS) (Figure 2) is a chronic and prevalent functional GI disorder (FGID), with recurrent abdominal pain and changes in bowel habit as its characteristic and defining symptoms.[47,48] Although the most typical symptoms of IBS are attributed to abnormal functioning of the lower GI tract, patients with IBS are often known to suffer from other symptoms, including psychological distress such as anxiety, depression, and somatisation.[47] Thus, IBS and other FGIDs are currently described as disorders of an altered gut-brain interaction.[49,50] IBS is more common in women and in younger adults (<50 years of age).[49] While the worldwide prevalence of IBS varies depending on the country and the diagnostic criteria used, recent studies estimate a 4.1% - 4.8 % prevalence based on the current Rome IV criteria.[51-53]

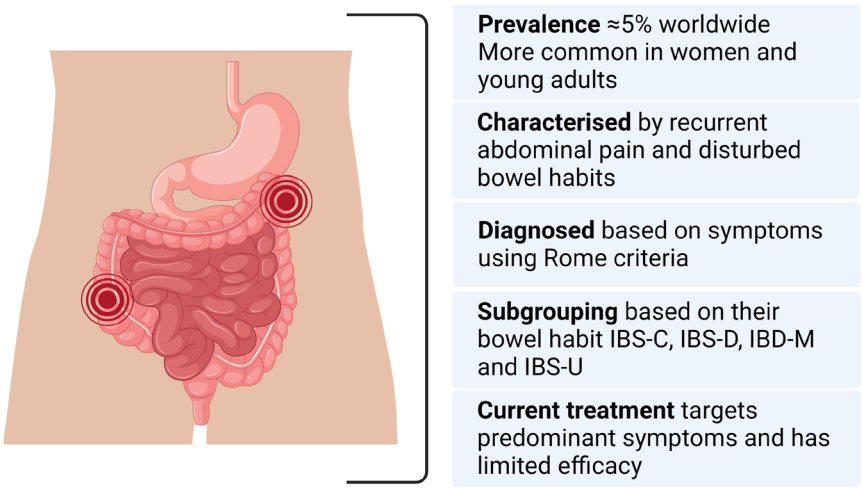

Figure 2. Overview of IBS. *IBS is a common FGID characterised with chronic abdominal pain, associated with change in bowel habits. Despite its high prevalence, this multifactorial disorder remains incompletely understood and effective treatment options are currently limited. Created with BioRender.com.*

Although not life threatening, the chronic remitting-relapsing nature of IBS symptoms considerably diminishes the quality of life and results in significant global health care costs.[51] Despite the progress made in the last decade to understand this multifactorial disorder, its pathophysiology remains incompletely understood and effective

treatment options are limited.[54] Thus, further studies evaluating the pathophysiology and treatment options for IBS play an important role towards reducing the socioeconomic effects of this disorder.

DIAGNOSIS OF IBS PATIENTS

Traditionally considered as a disorder with no obvious structural or biochemical abnormalities to explain its symptoms, or confirm diagnosis,[47] continuously evolving evidence however advocates distinct pathophysiological disturbances, which are further discussed in the following sections. Nevertheless, with no well-defined indicative biomarkers, current diagnosis of IBS is based on clinical history and symptom assessment using the Rome criteria, where the current symptoms must be present for at least 3 months, with onset 6 months prior to diagnosis.[49,55-57]

Alongside a positive symptom-based diagnosis using the Rome criteria, the medical history and absence of alarm symptoms, such as weight loss and rectal bleeding are important in the initial evaluation of IBS.[47,48] Additionally, several routine tests, such as blood tests including complete blood count, C-reactive protein and coeliac serology, as well as stool tests including faecal calprotectin are used to exclude other organic GI diseases, such as inflammatory bowel disease (IBD) and coeliac disease,[55,58] and normal results confirm an IBS diagnosis.[47,55]

The Rome criteria, updated from Rome III to Rome IV in 2016, is the current gold standard for clinical diagnosis of IBS.[57] The IBS patients in the cohorts included in this thesis, recruited prior to 2016, were however diagnosed according to the Rome III[56] (2006) criteria (**Paper I - III).** Table 1 summarises the main diagnostic criteria for Rome III and Rome IV.

The main difference in Rome IV compared to the previous Rome III version, is the removal of the term “discomfort”, leaving “abdominal pain” as the main diagnostic symptom. The rationale for this change was due to the unclear description and understanding of the word “discomfort” in different parts of the world.[57] Rome IV update also increased the symptom frequency threshold to at least 1 day per week compared to the previous requirement of at least 3 days in a month, making the criteria more stringent.[56-58]

***Table 1**. Rome criteria for IBS diagnosis*

Rome III[56] (2006)	**Rome IV**[57] (2016)
Recurrent abdominal pain or discomfort, 3 days per month in the last 3 months (12 weeks), associated with ≥2 of the criteria below: • Improvement with defecation • Onset associated with a change in stool frequency • Onset associated with a change in stool form	Recurrent abdominal pain, on average at least 1 day per week in the last 3 months, associated with 2 or more of the following criteria: • Related to defecation • Associated with a change in frequency of stool • Associated with a change in stool form

As a consequence of this update, the prevalence rates of IBS have decreased to approximately 4.5% using the Rome IV criteria,[51,53] which is less than half of the pooled global prevalence of approximately 11.2%, using the previous Rome criteria.[59] A global study however showed a prevalence of 40% for the pooled FGIDs.[51] This suggests that the shift away from IBS might be due to a change in diagnosis from IBS to other FGIDs, in particular functional constipation and functional diarrhoea, as an implication of the more restrictive Rome IV criteria for IBS.[51,53] However, this shift in diagnosis was shown to not have a major impact in western gastroenterology clinics with at least 85% of patients with IBS diagnosis according to Rome III also fulfilling the Rome IV diagnosis criteria.[60] This percentage was found to be somewhat lower, 61.6%-87.4%, based on the abdominal pain cut off in another comparison study.[61] Still, the majority of Rome III patients fulfil the more stringent Rome IV criteria. Nonetheless, the IBS patients fulfilling the Rome IV criteria have more severe clinical symptoms, including higher abdominal pain scores, and may compose a more "specific" population. This has been suggested to be of more importance in the research setting, allowing for recruitment of less heterogenous patients into clinical studies, rather than having a huge impact on treatment and patient care in clinical practice.[51]

SUBGROUPING OF IBS PATIENTS

IBS is recognised as a heterogenous disorder, where current treatment options commonly target the individual predominant symptoms.[54] Thus, subgrouping of IBS patients is commonly carried out in clinical practice for recommending treatment based on the predominant symptoms, as well as used for research purposes. In this regard, IBS patients are commonly divided into subtypes based on their bowel habits.[56,57] As per the Rome III criteria (used in the studies included in this thesis, **Paper I – III**) IBS subtypes are based on Bristol stool form (BSF) scale (Figure 3) characteristics. There are four main subtypes, namely IBS with constipation (IBS-C), IBS with diarrhoea (IBS-D), IBS patients with mixed bowel habits or mixed IBS (IBS-M), and un-subtyped or unclassified IBS (IBS-U).[56] The BSF scale grades the stool form using 7 ordinal categories (Figure 3).[62] The subtyping is based on predominant bowel form or consistency and percentage of that bowel form at a particular point in time (Table 2).

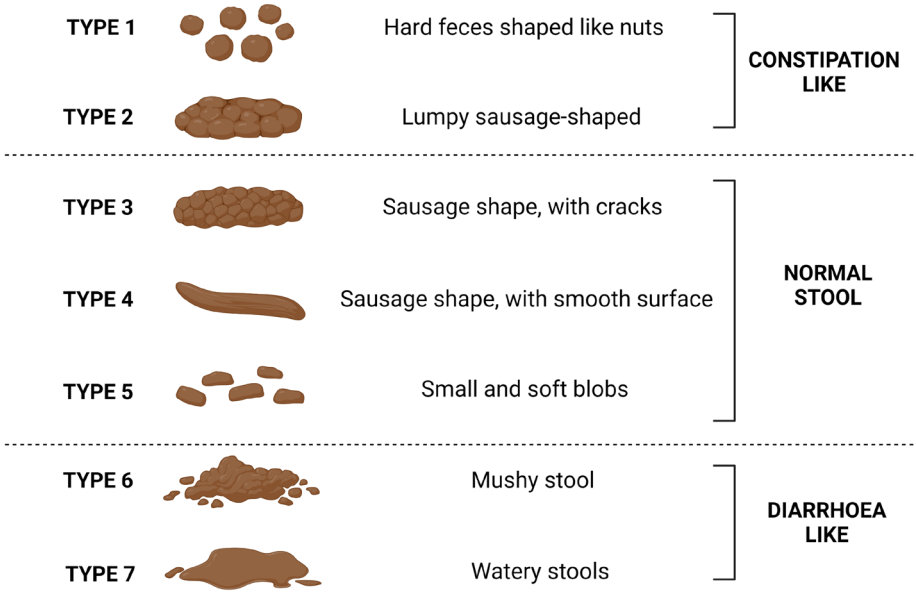

Figure 3. Bristol stool form scale. *The seven types of stool forms used for subtyping IBS patients based on bowel habit. Types 1,2 with 'constipation like' and Types 6,7 with 'diarrhoea like' are regarded as abnormal bowel habits. Created with BioRender.com.*

***Table 2**. Bowel habit based IBS subtyping according to Rome III criteria*[56]

IBS subtype	BSF type 1 or 2	BSF type 6 or 7
IBS-C	>25%	<25%
IBS-D	<25%	>25%
IBS-M	>25%	>25%
IBS-U	<25%	<25%

Note: Subtyping based on total stools recorded in a daily diary for at least 14 consecutive days and carried out in the absence of use of anti-diarrhoeal or laxatives.[56]

The classification into subtypes differs slightly amongst the Rome criteria. The Rome IV criteria subtyping are based solely on the days with at least one abnormal bowel movement, as opposed to the total number of bowel movements used in the Rome III version.[56,57] This subtyping based on bowel habit, though commonly used, is known to be erratic and overlapping.[63] IBS patients often switch from one subtype to another, with fluctuating and varying symptoms over time.[63,64]

The need for identification of other IBS subgroups based on underlying pathophysiology or symptom pathogenesis,[54] has generated some unvalidated hypotheses, where subgroups of patients have either a predominant biological or a predominant psychological abnormality.[65] Subgrouping of patients has also been investigated with mixed models using combinations of GI symptoms and psychological profile,[66-68] however these are yet to achieve validation for use in clinical and research settings.

For purpose of research, along with subtypes based on bowel habit, the studies included in this thesis (**Paper I – III**) also included subgrouping of patients based on symptom severity using the IBS Severity Scoring System (IBS-SSS).[69] IBS severity subgroups were based on validated cut-off scores for IBS-SSS, where mild IBS was defined as IBS-SSS < 175; IBS-SSS of 175-300 defined moderate IBS; and severe IBS was defined as IBS-SSS > 300.[69] Furthermore, IBS subgroups according to different levels of psychological distress were defined based on validated cut-off scores for Hospital Anxiety and Depression (HAD) scale.[70] IBS patients with anxiety or depression, including both borderline and clinically significant cases, were identified with HAD score $\geqslant$ 8, and patients without anxiety or depression, were recognised by HAD score < 8.

PATHOPHYSIOLOGY OF IBS

Despite its high prevalence and extensive evaluation, the underlying pathophysiology of IBS is still poorly understood. With IBS symptoms varying among different individuals, it often remains unclear which of the pathogenic factors trigger or augment the disease. Further, the cause and effect of the multiple factors cannot be disentangled.[68] However, the bidirectional dysregulation of the gut-brain interaction is well established and accepted as a disease model for IBS.[71,72] This is supported by the functional and structural alterations seen at multiple sites along the gut-brain axis, including an altered autonomic and central nervous system,[73] abnormal gut epithelium and permeability,[31] altered immune system,[74-76] and an altered intestinal microenvironment.[31,77]

IBS is currently described as a disorder of a disturbed gut-brain interaction with a heterogeneous and complex disease pathophysiology. Although defined as a disorder of the gut-brain interaction, it remains unclear if IBS begins in the gut, in the brain, or in both.[78] In parallel with the alteration in the gut-brain axis, and making matters even more complex, it has become increasingly apparent that the gut microbiota or gut microenvironment as a combined entity constitutes a critical element which plays an important role in health and disease.[71] Any alterations in the complex crosstalk between the host, the mucosal immune system, the nervous system, and gut microbiota, collectively known as the microbiota-gut-brain axis, plays a vital role in symptom development in IBS patients and hence is of importance for the pathogenesis of IBS.[50,79]

Among the recently recognised factors contributing to this multifactorial disorder, increased permeability,[31] altered immune system function and a low grade intestinal inflammation,[31,75,76] as well as an altered intestinal microenvironment[31,77] (Figure 4), are relevant for this thesis and will be further discussed. Several other factors outside the focus of this thesis, but well described as being important for symptom generation in IBS patients, include visceral hypersensitivity[80] regarded as one of the hallmarks of IBS, increased psychological distress,[81,82] altered GI motility[83] and altered GI secretion.[84]

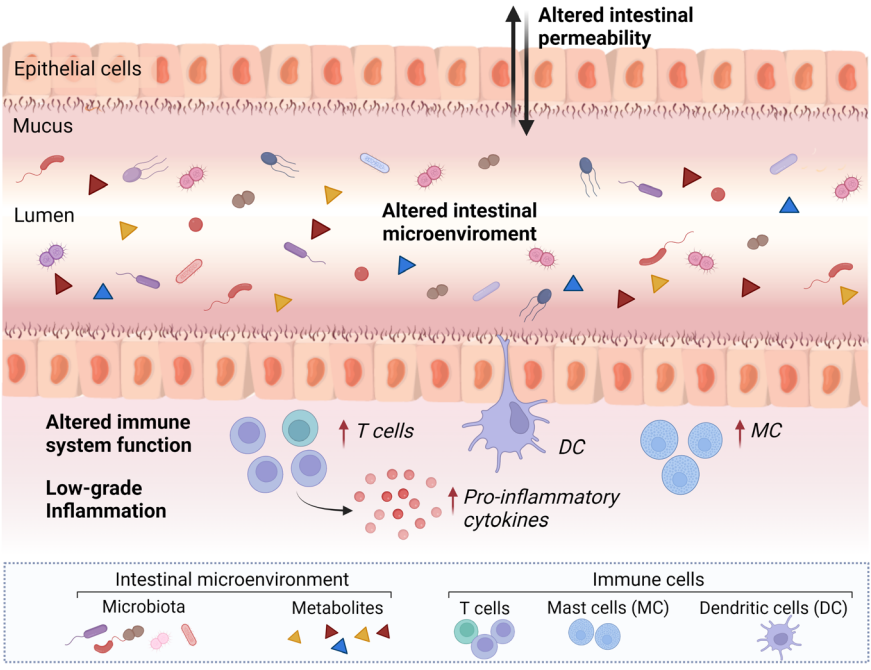

Figure 4. Overview of the pathophysiological factors of IBS covered in this thesis. *IBS is a disorder of a disturbed gut-brain interaction with a heterogeneous and complex disease pathophysiology implicating altered GI permeability, immune activation, and an altered gut microenvironment. Created with BioRender.com.*

Altered immune system function and low-grade inflammation in IBS patients

A previous bout of infectious gastroenteritis is established as one of the strongest risk factors for developing IBS (post-infection IBS), and hence supports the involvement of an altered immune system and low-grade immune activation in the pathogenesis of IBS.[76,85] As mentioned previously, multiple factors have been associated with disease and symptom generation in IBS, but none of these are present in all patients with IBS. In this regard, IBS, at least in a sub-population or subset, has been associated with immune activation characterised by changes in both the innate and adaptive immune system, including increased immune cell counts, particularly mast cells and T cells, as well as elevated levels of proinflammatory cytokines.[74-76,86]

Concerning innate immune cells, mast cells involved in several physiological functions, including wound healing, maintenance of homeostasis and defence against pathogens, are the most studied immune cells in IBS and have been implicated in its pathogenesis.[74,85,87,88] While there is evidence of increased mast cell numbers and their enhanced activation status in the intestinal mucosa of IBS patients,[87,88] the data is inconsistent. More relevant than the augmented mast cell numbers is probably the increased release of mast cell mediators, such as histamine and tryptase, with known effects on activation of enteric and extrinsic neurons, seen in the colonic mucosa of IBS patients.[88] Moreover, the proximity of mast cells to nerves in the colonic mucosa suggests the association of the nerve-mast cell interactions with the development of abdominal pain in IBS patients.[85,87] In addition to mast cell, other innate immune cells including monocytes, macrophages, and their proinflammatory cytokines, such as IL-6 and IL-8, which are known for their role in inflammatory response to pathogenic agents and modulation of the adaptive immune system, have also been implicated in the pathogenesis of IBS.[76,85] More recently, toll-like receptors (TLRs), the pattern recognition receptors which play a key role in mucosal innate immunity response and microbial tolerance, specifically TLR4, TLR5, TLR7 and TLR8, have also been shown to be dysregulated in patients with IBS.[89] These alterations together point towards microbial composition of the gut as a potential contributor to the low-grade inflammation in IBS patients.[89]

Furthermore, an activated adaptive immune system, reflected by increased numbers of T cells in various compartments of the intestinal mucosa in IBS patients, is implicated in its disease pathogenesis.[74-76,85,86,88,90] $CD4^+$ T helper cells, the most common of the T cells, have been reported to be increased in the colon in IBS patients.[75,86,90]. Along with the augmented numbers, the frequency of activated T cells, demonstrated by an increased frequency of T cells expressing activation marker CD25 (α chain of the IL-2 receptor), is also raised in patients with IBS, seen both in the colonic mucosa[88] as well as among the blood T cells[75]. Additionally, $CD4^+$ T cells expressing the gut homing marker integrin α4β7 and the lymph node homing marker CD62L, supportive of immune activation in IBS patients is also reported to be increased.[90,91] Although studies are not in agreement regarding the cytokine profile in IBS patients, an altered or imbalanced cytokine profile with elevated serum cytokines,[92] in particular enhanced

proinflammatory cytokines, including TNF-α, IL-1β and IL-6,[86] and decreased anti-inflammatory cytokine IL-10,[93] has been described in at least a subgroup of patients with IBS.[94,95] Altogether, evidence mounts towards the importance of immune activation and low-grade inflammation in the pathogenesis, at least in a subset of IBS patients.

As described previously, the GI tract with its complex interaction between the intestinal epithelial barrier, the mucosal immune system and the gut microenvironment, maintains balance between protecting against pathogens and developing tolerance to food and self-antigens.[4] This balance is lost in IBS and despite the considerable impact of the altered immunological function on symptom and disease development in IBS patients,[31,76] the mechanism leading to the aberrant immune activation described above remains incompletely understood. Among the suggested mechanisms, an altered intestinal permeability associated with a decreased expression of the tight junction protein zonula occludens,[96] and potential alteration in the mucus composition has been observed in IBS patients,[76] especially in IBS-D and post-infection IBS. Along with the structural alterations in the intestinal epithelial barrier, an increase in intra-epithelial lymphocytes is also associated with the intestinal mucosa of patients with IBS.[97]

Intestinal barrier dysfunction as a cause or consequence in patients with IBS, although remains in debate, appears to be of importance in the immunopathogenesis of IBS. The alterations in immune cell populations and inflammatory markers have directed the attention towards the immune system as a potential target and mode of action for therapeutic options for patients with IBS (**Paper IV**). Furthermore, the gut microbiota, being important in the development and regulation of the host mucosal immune system, is deemed to be of relevance in the altered immunological function in IBS and hence in the pathogenesis of IBS.[76,85]

Altered intestinal microenvironment in IBS patients

The intestinal microbial communities, their metabolites and other luminal components altogether make up the intestinal microenvironment. The intestinal microenvironment and its interactions with the host are not only necessary for immune homeostasis and gut health, as described in the previous section

"*Gastrointestinal tract – intestinal microenvironment*", it also influences the susceptibility of the host to gut related diseases and disorders including IBS.[98,99]

Gut microbiota alterations in IBS patients

Mounting evidence supports the role of an altered gut microbiota in the pathogenesis of IBS. This is particularly evident in the post-infection subset of IBS patients,[99] as well as from the increased risk of developing IBS associated with the use of broad-spectrum antibiotics.[100] Moreover, the alteration in composition, diversity and richness of gut microbiota observed in at least a subset of IBS patients has been reported in multiple studies.[79,101,102]

While several studies have identified differences between the microbiota composition of IBS patients compared to healthy subjects,[99,103-105] there is considerable overlap between the two groups and no distinct IBS profile has been identified. Supporting previous studies,[77,99] we described **(Paper I)** a distinct gut microbiota in IBS patients with a decreased abundance of *Clostridium* sp., *Bifidobacterium* spp. and *Faecalibacterium* spp. along with an increased abundance of *Bacteroides fragilis*, as compared to healthy subjects. Although not entirely consistent with existing data, our study also showed change in the abundance of Firmicutes-associated taxa Verrucomicrobia, in particular *Akkermansia muciniphila* and *Eubacterium* spp., considered as health promoting microorganisms[106] **(Paper I).** Alterations in these bacterial taxa are deemed to be associated with the distinct gut microbiota in IBS patients (Figure 5).

In line with other research,[107,108] our study demonstrated subtype-specific variations in microbiota composition, particularly between the bowel habit-based subtypes IBS-C and IBS-D. This variation, seen as the elevated abundance of Firmicutes, *Escherichia* spp. and *Shigella* sp. and *Mycoplasma homini* in IBS-C patients compared with IBS-D patients, highlights the association of specific bacterial taxa with bowel habit in IBS patients **(Paper I).** Our study, possibly owing to the sampling site, failed to detect association between a distinct gut microbiota profile with other gastrointestinal and psychological symptoms in IBS patients (**Paper I**). Other studies have however been able to associate alterations in the microbiota composition with a range

of IBS symptoms and symptom severity,[104,105,109] as well as with intestinal immune response,[105] further supporting the central role of gut microbiota in the symptom development and pathophysiology of IBS. While the majority of the existing data on gut microbiota composition in IBS patients (including **Paper I**) have been evaluated using faecal samples, owing to its convenient accessibility, mucosa-associated microbiota which is known to be different in composition from the faecal microbiota,[105] also deviates IBS patients compared to healthy subjects.[105,107,108] Further, mucosal microbiota has been proposed to be more related to symptom generation and severity of symptoms in IBS patients.[105] Regardless, both types of samples are of relevance as both faecal and mucosal microbiota, due to their proximity to host cells including the nerves and immune cells, can affect the host via host–microbial interactions and subsequently influence IBS symptoms.[107]

Even though association is not always causation, the decreased abundance of *Clostridium* sp., *Bifidobacterium* spp. and *Faecalibacterium* spp. in IBS patients (**Paper I**), being important butyrate-producing and anti-inflammatory organisms and playing an important role in maintaining gut mucosal health, are promising probiotic treatment targets for ameliorating IBS symptoms and are discussed further in this thesis (see section *Prebiotic and probiotic treatment*). Apart from its role in disease pathogenesis and as inducers of gut symptoms, gut microbiota composition may also hold the potential of being a predictor of clinical responsiveness to dietary interventions.[110-112] This predictive potential of faecal microbiota profiles was also deliberated in our study (**Paper II**) and will be described further.

The heterogeneity between studies, IBS diagnosis criteria and culture techniques used, as well as heterogeneity of the microbiota in IBS patients and its considerable overlap with healthy subjects have led to inconsistent and sometimes contradictory findings between various studies. Further, the possible influence of GI transit on the gut microorganisms in various IBS subtypes has made it difficult to determine any specific bacterial taxa or microbiota profiles correlated to IBS patients and subtypes of IBS.[77,101,107] Nevertheless, the accumulated results confirm an altered gut microbiota composition in IBS patients and its importance in IBS pathogenesis.

Alterations in gut metabolite production in IBS patients

While previous research has focused mainly on gut microbiota, mounting reports on the effects of intestinal microbial metabolites on human health has highlighted the role of metabolites.[32] To understand the mechanisms by which the gut microbiota drives disease and symptom pathophysiology in gut disorders and diseases, metabolite composition as a functional output or a snapshot into the complex host-microbial interaction might be a superior tool.

Alterations in metabolite production in the gut, from either the microbiota or the host and their interaction, reflecting the functional status of the intestinal microenvironment, have been implicated in the manifestation of IBS symptoms.[113] Specific classes of metabolites, notably amino acids, in particular tryptophan metabolites, and others such as SCFAs, bile salts and purines, have previously been implicated in the pathogenesis of IBS.[78,107,113-115] Along the same line, our study (Figure 5) depicted alterations in the faecal metabolite profile of IBS patients, characterised by an increase in several amino acids and amino acid metabolism intermediates such as alanine, glycine, proline, phenyl pyruvic acid and pyruvic acid (**Paper I**). An increase in purine metabolites, hypoxanthine and xanthine, was also seen in IBS patients compared to healthy subjects. These metabolites have been implicated in several biological pathways and functions, including intestinal barrier development and immune response and inflammation.[114,116,117] Hence, alteration in these metabolites, as well as several amino acid uptake pathways, predicted to be differentially regulated in IBS patients compared to healthy subjects, further advocates the importance of amino acid and purine metabolism pathways in the disease pathophysiology of IBS (**Paper I**). Another noteworthy alteration seen in our study (**Paper I**), was the increased abundance of tryptophan, an intermediate metabolite in serotonin metabolism, known for its role in intestinal immune responses and intestinal barrier integrity.[118-120] Serotonin, a neurotransmitter, plays a key role in the enteric nervous system and is also shown to be involved in the regulation of secretion and motility in the gut.[121] Altered serotonin metabolism has also been associated with IBS, however the studies are limited and contradictory.[122] The altered levels of tryptophan, along with increased levels of other amino acid metabolism metabolites, such as glycine, glutamic acid and ornithine seen in our study (**Paper I**), which are also molecules related to neuro-immune signalling pathways and are associated with IBS-specific brain changes,[123] further support the

importance of metabolites in the aberrant microbiota-gut-brain crosstalk implicated in the pathophysiology of IBS.

Despite the heterogeneity of IBS patients, distinct faecal metabolite profiles have been described in IBS patients,[114] with variations in metabolite profiles also correlated with specific IBS-subtypes based on bowel habits[107] (also seen in **Paper I**), other GI symptoms and symptom severity, as well as psychological symptoms in IBS patients. The overall distinct intestinal metabolite profile of IBS-D patients described in our study with altered tryptophan and putrescine levels (**Paper I**) is also consistent with the current available literature.[107,115,124]

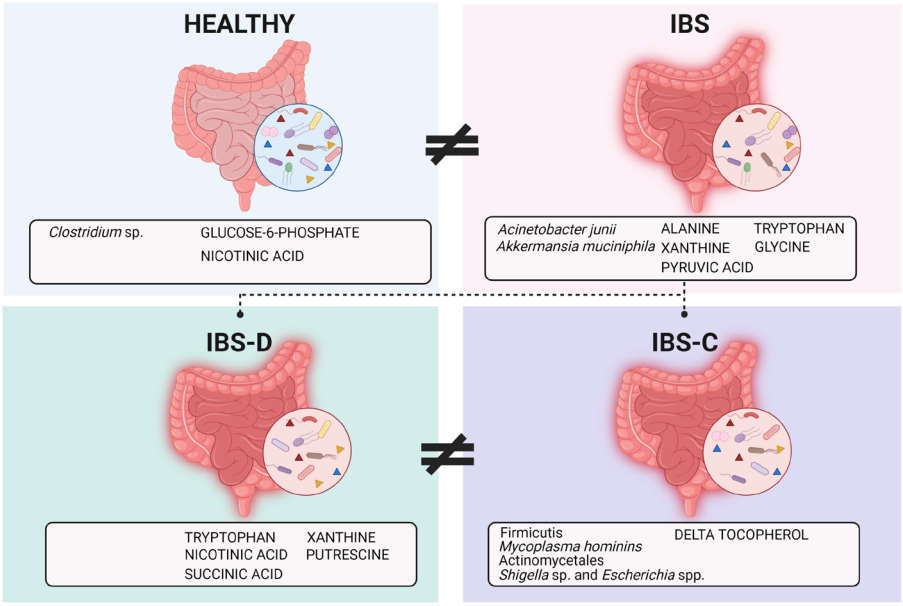

Figure 5. The distinct intestinal microenvironment in patients with IBS and IBS subgroups compared to healthy subjects (Paper I). *The intestinal microenvironment defined by the combined faecal microbiota (n = 54) and metabolite (n = 155) composition is altered in IBS patients, as compared to healthy subjects. The intestinal microenvironment could also differentiate between IBS patient subtypes based on bowel habit, in particular IBS-C and IBS-D. Specific microorganism and metabolites found in our study to be most associated with IBS and IBS subtypes, as well as with healthy patients are listed in a box associated with each group, respectively. Created with BioRender.com.*

Alteration in the integrated microbiota-metabolite profiles

As described above, numerous studies have previously used either gut microbiota[77,103-105,108] or recently metabolite profiles[113,125] to separate between IBS patients and healthy subjects. Considering the complex microbiome-gut-brain interaction it would however be beneficial to integrate several biological and clinical factors to study gut related diseases and disorders like IBS.[107,126] Along these lines, our study showed that when combining the data from faecal microbiota and metabolite profiles, the distinction between IBS patients (including all subtypes) and healthy subjects substantially improved (Figure 5) (**Paper I**). This joint classifier, in agreement with a limited number of other studies,[115,126] extends our understanding into the potential mechanisms underlying the host–microbiome interaction in IBS. A recent study also showed association between the integrated microbiota-metabolite profiles identified for IBS patients with clinical features such as stress response related to GI symptoms.[126] Another recent study by Mars et al.[107] further suggests the integration of several longitudinal multi-omics analyses including gut microbiome, metabolome, host epigenome, and transcriptome to enhance our ability to identify functional mechanisms that add to the potential pathophysiological mechanisms implicated in IBS.

Along with providing insight into disease mechanisms, intestinal microenvironment also offers potential biomarkers to be used as future non-invasive diagnostic tools for potential use in a clinical setup.[127] Moreover, the intestinal microenvironment in particular gut microbiota, owing to its complex crosstalk with the intestinal barrier, mucosal immune function, and host diet, offers new therapeutic targets for IBS patients. In line with this, therapeutic strategies aimed at modulating the intestinal microbiota and in turn the intestinal microenvironment, such as prebiotic and probiotic treatments appear to be promising treatment options for patients with IBS and are discussed further below.

Altogether, an altered intestinal microenvironment composition and function, which could be either the cause or a consequence of IBS, plays a key role in the pathogenesis and pathophysiology of IBS. This alteration, which yet is incompletely understood, needs a better description, and remains a challenge for the future.

MANAGEMENT OF IBS SYMPTOMS

Although IBS is characterised by recurrent abdominal pain and changes in bowel habit,[47,48] patients often suffer from several other GI related symptoms, such as bloating and abdominal distension, as well as multiple extra-intestinal symptoms, including psychological distress, headaches, back pain and sleep disturbances, amongst many others.[65,128] Hence, the biggest challenge in the management of IBS, besides its incompletely understood pathogenesis and pathophysiology, is the diverse range of symptoms and the fact that the predominant symptoms also vary substantially from one patient to another.[129]

The main goal of treatment of IBS is to relieve symptoms and for successful management of IBS symptoms, a stepwise approach is usually advocated. Starting with a confident diagnosis, clear information and patient education, and establishment of a good physician–patient relationship is considered crucial for good management.[129,130] General advice on diet, lifestyle and physical activity are also included in the first stage of management.[129] A majority of IBS patients associate their symptoms, particularly abdominal bloating and pain, to be triggered by ingestion of a wide range of foods,[98] leading patients to frequently make dietary adjustments often without professional help and succumbing to an inadequate diet.[131,132] Thus, the general advice is usually followed up with dietary education and depending on response of patients, specific dietary advice as well as dietary interventions and the addition of prebiotic and probiotic supplementations may be considered.[129] These are further discussed in the sections, *Dietary modifications* and *Probiotic and prebiotic treatment* below.

In case the traditional management advice does not help to reduce IBS symptoms, the next line of management includes pharmaceutical treatment. Since no treatment cures IBS, and relief of symptoms is the best that can be accomplished, the pharmaceutical treatment is based on the predominant symptoms.[47,129] In this regard, the first line of targeted therapy most commonly used for IBS-D is loperamide, for IBS-C the use of fibre or osmotic laxatives is usually recommended as the first option, whereas antispasmodics and tricyclic antidepressants are the most widely used pharmacological treatments for the management of pain in IBS patients.[47,54,129] Since the majority of IBS patients have more than one predominant symptom, a multi-integrated approach

which consists of combining treatments including the use of psychotropic drugs and psychological treatment options is shown to be more effective.[129] However, efficacy of most drugs and first line therapies is considered to be modest[133-135] with risk of adverse effects, and since they target individual symptoms and not the overall disease burden, a substantial proportion of patients are refractory to the current limited treatment options.[129] It is thus not surprising that IBS patients often turn to alternative approaches.

Targeted therapies are still in evolution and considering the role of food and gut microenvironment as relevant factors involved in symptom generation in IBS, it seems logical that modulating these factors might be promising treatment options for these patients. Owing to the heterogeneity of IBS patients, it might also be worthwhile to identify subgroups of IBS patients who are more likely to respond to specific therapeutic choices. Since no single treatment fits all in the case of management of IBS symptoms and available pharmacological treatments have modest efficacy with risk of adverse effects, more alternative treatment strategies for patients with IBS are desirable. The safety, low cost and the wide range of beneficial effects favours the further evolution of therapies such as dietary modifications, probiotics, prebiotics, as well as use of medicinal foods and herbal products such as *Aloe barbadensis* Mill. in the management of IBS symptoms.

DIETARY MODIFICATIONS

Majority of IBS patients identify eating or ingestion of specific food items to be the principal trigger of their GI symptoms.[136,137] While the mechanisms of food intolerance in IBS remain unclear, the role of dietary factors in the pathogenesis and symptom development is becoming increasingly recognised.[98,138] Hence, making dietary manipulation an attractive treatment strategy, where specific food components thought to trigger or worsen symptoms, are avoided for a given period of time and then progressively re-introduced to evaluate the effects on symptoms,[138] followed by a personalised and less restrictive maintenance diet.[139]

Among dietary interventions, there is little evidence for the efficacy of the gluten-free or lactose-free diet in IBS patients without lactose intolerance or coeliac disease, and these are usually not recommended to patients.[129] On the other hand, growing evidence suggests the

effectiveness of the low fermentable oligo-, di-, and monosaccharides and polyols (FODMAP) diet in managing IBS symptoms and improving quality of life in IBS patients.[140] The use of low FODMAP diet is widely accepted, in agreement with its symptom reducing effects supported by several studies.[141,142] Still, its superiority to conventional dietary advice, which focuses on avoiding large meals, fat, insoluble fibre, caffeine, and gas-producing foods such as beans, has however not been clearly established.[143] The low FODMAP diet is based on reducing the intake of small, indigestible, and fermentable carbohydrates including fructans, galacto-oligosaccharides (oligosaccharides), lactose (disaccharide), fructose (monosaccharide), and mannitol, sorbitol, maltitol and xylitol (polyols). It has been demonstrated to reduce water flux into the intestinal lumen through osmosis, gas production due to fermentation, and thus associated with reduction of intestinal distension and GI symptoms in IBS patients.[141,143,144] However, the low-FODMAP diet being restrictive, eliminates certain food items commonly regarded as healthy prebiotics and clashes with the prebiotic concept (See section *Probiotic and prebiotic treatment* for more detail) and thus risks perturbations in the balance of the gut microbiota.[145] In this regard, a low-FODMAP diet has been associated with reduction in health-associated bacteria, such as *Bifidobacterium*, and a shift in the gut microbiota composition.[146] Given the important role of the gut microbiota for intestinal and general health, these results suggest negative consequences and caution for the long-term use of dietary interventions such as the low FODMAP diet, since this diet can reduce the number of beneficial gut bacteria.[147] Therefore, another suggested approach is to combine this dietary intervention with a probiotic preparation,[148] as well as safe prebiotic or prebiotic-like options[149,150] to improve efficacy and compensate for the reduction of beneficial *Bifidobacterium* spp. and altered gut microbiota.

Altogether, the low FODMAP diet is complex, challenging and requires supervision by a qualified dietitian. Further, given that only a proportion of patients with IBS respond to the low FODMAP diet,[151] as well as other dietary interventions, and keeping in mind their potential detrimental long-term effects, there is a need to understand how these diets work, as well as to identify biomarkers that predict response.[112,152] Hence, the potential role of the gut microbiota and metabolite profiles in predicting the efficacy of dietary interventions like the low FODMAP diet, prior to starting the intervention, may be an asset,[110-112,153,154] although still needing further research and validation.

Apart from exclusion diets, therapeutic strategies for patients with IBS have also for decades included the use of dietary fibres, particularly for constipation-predominant IBS (IBS-C), as bulking agents.[155] Dietary fibre has the potential to change microbial diversity and richness, stimulate the growth and/or activity of beneficial bacteria, such as *Bifidobacteria* or *Lactobacilli*, produce beneficial metabolites like SCFAs and promote gut health.[156] However, recent studies suggest that while certain fibres may be beneficial, others exacerbate symptoms such as bloating, flatulence and abdominal cramps and therefore limit their usefulness.[157,158] Hence, the role of fibre in patients with IBS is formulation-dependent, requiring further research and validation.[159] The role of specific nondigestible carbohydrates referred to as prebiotics, recently also referred to as "microbiota accessible carbohydrates" is discussed in the next section *Prebiotic and probiotic treatment*.

PROBIOTIC AND PREBIOTIC TREATMENT

Considering the evident role of gut microbiota in human health and the well accepted altered gut microbiota composition/function in IBS patients, with its relevance in IBS pathogenesis, the use of therapeutic interventions that could beneficially modulate the microbiota composition and / or its functional capacity and improve symptoms have increasingly evolved. Among these, supplementation with probiotics and prebiotics are of relevance to this thesis.

Probiotics in IBS

Probiotics, defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host"[160] by the International Scientific Association for Probiotics and Prebiotics, usually include well defined species of bacteria or yeasts, in particular from the genera *Lactobacillus*, *Bifidobacteria* and S*accharomyces*.[99,160-162] Commonly used probiotics consist of either single strains, or mixtures of strains, and are available in different forms for human consumption.[160] A vital task for the probiotic strain(s) is surviving transit through the GI tract and imparting benefit to the host by influencing the hosts' microbial ecosystem, immune function and/or colonic fermentation.[163]

Probiotics have been demonstrated, as either specific species or strains, or combinations, to have beneficial effects in improving global IBS symptoms and abdominal pain.[54,99,163] Abundance of the health-related bacterial species *Bifidobacterium* and *Lactobacillus,* often reported to be altered in patients with IBS,[102] have also been associated with improvement of global IBS symptoms.[54,161,162] In line with this, interventions using specific strains of *Bifidobacterium bifidum*,[164] *Bifidobacterium longum*,[165] *Bifidobacterium infantis*,[166,167] *Bifidobacterium lactis*,[168] *Lactobacillus acidophilus*,[169] and *Lactobacillus plantarum*,[170] have all been reported to alleviate or normalise one or more IBS-related symptoms. The symptoms affected include abdominal pain, overall symptom severity, bloating, altered bowel habit as well as depression, and the interventions have been demonstrated to improve overall quality of life in patients with IBS. Results from recent clinical studies also support the long term usage and benefit of *Lactobacillus plantarum*,[171] and the role of *Lactobacillus plantarum* CCFM8610,[172] with improving gut microbiota diversity and modulating microbial dysbiosis, in particular in IBS-D patients. Further, while *Lactobacillus paracasei* HA-196 has shown efficacy in reducing GI symptom severity and improving psychological well-being of patients with certain IBS subtypes,[173] another strain of *Lactobacillus paracasei* CNCM I-1572 was reported to modulate gut microbiota and reduce immune activation in IBS, but without improving IBS symptoms.[174] In addition to individual probiotic strains, several multi-species and multi-strain probiotics have also been reported to be effective in alleviating symptoms in IBS patients and specific subgroups, as well as inducing alterations in the intestinal microbiota profiles.[175-177]

Numerous individual studies, beyond the scope of this thesis, have attested the overall beneficial, but modest, effects of probiotic interventions in IBS, without significant adverse effects, however, there are several inconsistencies.[178-181] These are most likely due to the heterogeneity of IBS patients, difference in bacterial strains, dosage, and design and duration of the interventions. Further, which particular strain or species that may be better, and the mechanism of action of individual probiotics remain unclear[161] and warrant additional clinical studies and further research.

Prebiotics in IBS

Prebiotics are an alternative therapeutic strategy which may be used to modulate the gut microbiota in the attempt to improve host health.[158] Nondigestible dietary carbohydrates such as inulin-type fructans, galacto-oligosaccharides and fructo-oligosaccharides, are commonly investigated prebiotics that act as "substrates that are selectively utilised by host microorganisms, conferring a health benefit".[158,182] They have been associated with stimulation of the growth and/or activity of bacterial species already resident in the colon such as *Bifidobacterium,*[158,183-185] increased production of faecal short-chain fatty acids, modulating lipid metabolism, as well as reducing gut-associated inflammatory markers.[158,186,187] Prebiotics also have a slight advantage over probiotics, in that they do not risk degradation during transit through the GI tract and their production is less complicated.[188]

Although the ability of prebiotics to modulate the gut microbiota provides a rationale for their use as a potential therapeutic strategy in managing symptoms in IBS, few studies have investigated the efficacy of these dietary ingredients in patients with IBS. Prebiotics such as trans-galactooligosaccharide (at a low dose),[188] beta-galactooligosaccharide,[150] inulin,[189] and short-chain fructo-oligosaccharides[190] have been reported to alleviate GI-related and non-GI-related symptoms in patients with IBS and IBS subgroups. Still, higher doses of inulin and fructo-oligosaccharides have been associated with side effects such as increased bloating, flatulence, or abdominal pain or have failed to affect symptoms favorably.[191-193]

Altogether, existing data on the beneficial effects of prebiotics on GI symptoms and quality of life in patients with IBS, although suggested to be dose dependent,[163] are inconsistent and remain unconfirmed.[161,191] However, the ability of prebiotics to modulate the composition of gut microbiota and stimulate beneficial bacteria such as bifidobacteria,[190,191] being an important factor in the pathogenesis of IBS, warrants more research. There is therefore a definite need to identify additional beneficial carbohydrates and functional foods with prebiotic properties that promote human gut health without any adverse symptoms or effects, as therapeutic options for ameliorating symptoms in IBS patients.

MEDICINAL FOODS AND HERBAL PRODUCTS

Due to limitations such as inadequate effectiveness, potential risks, and side effects as well as high cost of current conventional medicinal treatments for IBS, many patients are attracted towards the use of alternative treatment strategies such as medicinal foods and herbal products.[194,195] It has been reported that usage of such alternative treatments is greater for IBS than other GI disorders, and up to 50% of IBS patients are reported to take a recourse to these alternative therapeutic options.[196-198]

Among medical foods under development for the management of IBS, peppermint oil is the most researched. Although enteric-coated peppermint oil appears to be well-tolerated, with generally mild adverse effects, in the short-term management of IBS symptoms, such as relief of abdominal pain and discomfort,[194,199] its efficacy appears to be dependent on formulation and targeted release of the active ingredient in the small intestine.[200] Further, STW-5 a liquid preparation made from extracts of nine herbs, commercially available as Iberogast®, is associated with multiple mechanisms of action and relief of a wide range of GI symptoms, including reducing symptoms of functional dyspepsia.[201] One placebo-controlled study with positive effects of STW-5 on improvement of symptom severity in patients with IBS has also been reported.[201] Glutamine, asafoetida, curcumin, carmint, aniseed and fennel essential oils are some other medicinal food/herbal agents that have shown some evidence of efficacy in improving IBS symptoms. Nevertheless, well-designed clinical studies are still lacking.[54,194]

The role of medicinal foods and herbal therapies as a therapeutic option for patients with IBS remains unclear. However, these therapies may be considered as add-ons or complimentary therapies, in refractory patients working alongside drugs, in disease management to improve patient outcomes.[195] Owing to their promising efficacy and considerable safety, herbal products are usually used unsupervised, without consulting a doctor or determining the correct dosage. Along with the need for additional clinical studies for evaluation of efficacy, increased awareness among healthcare providers is also necessary for providing unbiased information about the use of these therapeutic options to IBS patients.

Medicinal foods and herbal products are complex by the virtue of their diverse active components, which are not fully identified and lack conclusive results about their mechanisms of action. The compositional complexity of herbal products with multiple mechanisms of action may be of benefit for the management of heterogenous symptoms of IBS. One such compositionally complex medicinal plant is *Aloe barbadensis* Mill., commonly known as Aloe vera or Aloe. It has a long history of use, is suggested to have immunomodulatory properties (**Paper IV and V**) and has also been associated with therapeutic effects in patients with IBS (**Paper II and III**) and being the focus of this thesis, it will be discussed in further detail in the next section.

ALOE

The genus *Aloe* comprising of over 400 species of perennial tropical plants belong to the Xanthorrhoeaceae family and Asphodeloideae subfamily, according to the updated Angiosperm Phylogeny Group APG IV system[202] (Table 3). *Aloe* derives its name from the Arabic word "Alloeh" and Hebrew word "allal" both meaning bitter substance,[203,204] and are known to have originated in the warm, dry climates of Africa. With the first human use of *Aloe* plants recorded back to the Mesopotamia civilisation ca 2200 BC, and its well-documented use in ancient Egypt, Greece, India and China, *Aloe* plants have enjoyed a long history of lay acceptance and been used for centuries as topical and oral therapeutic agents for their wound healing, skin care and laxative properties.[203-205] The most commonly studied *Aloe* species known to be associated with medicinal properties include *Aloe barbadensis* Mill., *Aloe perryi* Baker, *Aloe ferox*, and *Aloe arborescens*. Amongst these *Aloe barbadensis* Mill. or Aloe vera (Aloe) the most widely cultivated and most commercialised *Aloe* species, is the focus of this thesis. In chemical terms, CAS numbers used for *Aloe* and *Aloe barbadensis* Mill. are 8001-97-6 and 85507-69-3.[206,207]

Table 3. Taxonomic classification of Aloe vera[203]

Rank	**Scientific name**
Kingdom	Plantae
Phylum	Spermatophyta
Sub phylum	Angiospermae
Class	Monocotyledonae
Order	Asparagales
Family	Xanthorrhoeaceae
Sub Family	Asphodeloideae
Genus	Aloe
Species	*Aloe vera (*L.) Burm. f.
Synonyms	*Aloe barbadensis* Mill.
	Aloe vera L.
	Aloe vera
	Aloe humilis Blanco
	Aloe perfoliata var. vera L.,
	Aloe vulgaris Lam

ALOE BARBADENSIS MILL.

Aloe vera (L.) Burm. f. is the correct species name for the widespread *Aloe barbadensis* Mill., which is the most widely accepted species of the genus *Aloe*.[208] The plant is commonly known as Aloe vera, meaning the true Aloe. Although previously considered to be part of the Liliaceae family and until recently designated to the Aloeaceae family,[209] Aloe vera (Aloe) shares its updated taxonomical classification with other *Aloe* plants (family: Xanthorrhoeaceae, subfamily: Asphodeloideae (Table 3).

Aloe, considered to be medicinally potent with numerous beneficial attributes, is perhaps one of the most popular herbal remedies used today and can be found as an ingredient in a myriad of health and cosmetic products. It is cultivated worldwide in dry sub-tropical and tropical climates, with Mexico being the main producer, followed by South America, China, Thailand, Europe, and the United States.[203,205]

Aloe are succulents or xerophyte plants which are adaptable to grow with low water availability and have their green fleshy leaves, which possess extensive water storage tissue, in common with other *Aloe* species.[205] Another feature of these succulent plants is the use of crassulacean acid metabolism, an adapted photosynthetic pathway which involves malic acid production.[210]

Structural composition

Aloe plants grow as rosettes of tapering sword-like green fleshy leaves, which can grow to 60cm long and 10cm broad.[211] The plant can have around 12-16 leaves and matures around the age of 4 years, with a total lifespan of up to 12 years. Large Aloe plants flower giving bright yellow tubular flowers arranged in a slender loose spike.[203,208]

The Aloe leaf, the part of the plant implicated for its therapeutic properties, can be divided into three distinct parts[209,212,213] (Figure 5):

1. The ‘Rind’; the thick epidermis covering the leaf including the tip, bases, and thorns forming the outer green shield. The rind makes up 20-30% weight of the whole leaf and has thick cuticles made up of

multiple layers of cells with dispersed chloroplasts, where carbohydrates, fats and proteins are synthesised.

2. The 'Aloe latex' or the yellow exudate; sandwiched between the outer rind and the inner gel matrix, lies the yellow exudate which is stored and transported in the pericyclic tubules of the vascular bundles. The Aloe latex also commonly referred to as "Aloe juice" or "Aloe sap" is a bitter tasting liquid rich in anthraquinones and known for its laxative and cathartic properties.

3. The 'Aloe gel', 'Fillet' or 'Inner gel matrix' (**Paper II – V**); the innermost portion of the Aloe leaf is made up of thin-walled parenchyma cells which harbour the clear mucilaginous gel extract, called the Aloe gel, which attributes to a majority of the beneficial effects of the plant.[214] The Aloe gel represents the main portion of the leaf by volume, making up approximately 65-80% of the whole leaf. The Aloe inner gel matrix is the water and energy storage component of the plant, storing not only large amounts of water, but also all the excess carbohydrates, as well as minerals, and malic acid formed by crassulacean acid metabolism.

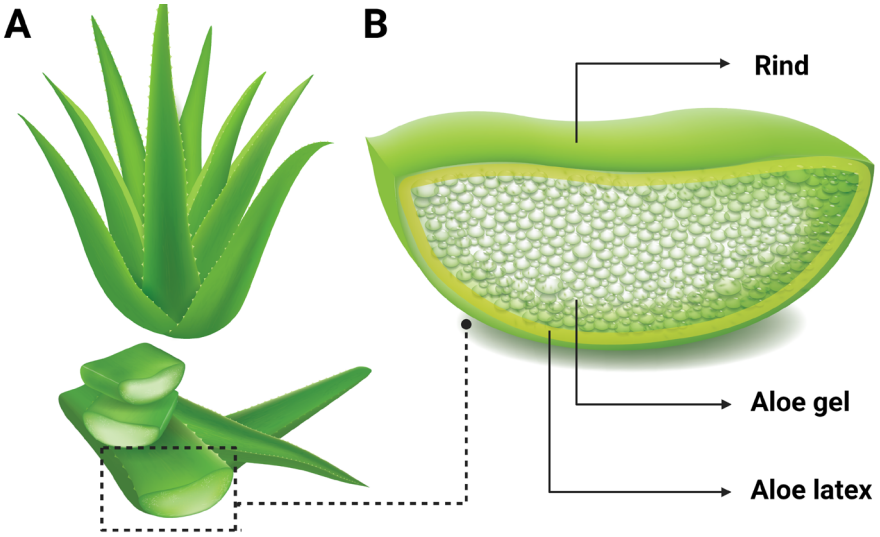

Figure 6. Schematic presentation of Aloe. *The Aloe plant and Aloe leaf with a cross-sectional view (A) depicting the three main structural components of the Aloe leaf (B). Created with BioRender.com.*

Chemical composition

The Aloe leaf is known to have a myriad of chemical components which are distributed in the Aloe latex and the Aloe gel, the two commercially important products obtained from the Aloe plant and implicated with numerous medicinal properties.[205,215,216] While the in-depth phytochemical composition of the Aloe leaf is beyond the scope of this thesis, it is described briefly with focus on the composition of the Aloe gel, being the source of the Aloe extracts studied in this thesis (**Paper II – V**).

Chemical Composition of Aloe latex

The Aloe latex or yellow exudate is a bitter tasting liquid stored and transported via the pericyclic tubules, sandwiched between the outer leaf and the inner gel matrix.[205,216] The yellow exudate, also referred to as Aloe juice (for more detail on terminology refer to section *Aloe commercial products and their quality control*), is the component of Aloe leaf which is implicated with its traditional intended use for the short-term treatment of occasional constipation, and is well described in several monographs and pharmacopeias.[203,208,217] Over 100 chemical compounds have been identified from the Aloe latex, which is largely phenolic in nature (summarised in Table 4), containing a series of glycosides known as anthraquinones or hydroxyanthracene, recognised as irritants to the GI tract and for their laxative properties.[205,216] The major phenolic compounds present in the latex are barbaloin (aloin (15-40%)), which occurs naturally as a mixture of two diastereomers, aloin A and B.[218] Aloin, is made up of sugar molecule D-glucose, bound to the anthracene ring by a β-glycosidic linkage giving a β-(1→10) C–C bond.[219] Other phenolic compounds include aloesin, aloeresin A and aloe-emodin.[218] Besides the phenolic compounds, the Aloe latex also contains small quantities of polysaccharides and free sugars such as glucose, as well as aromatic aldehyde compounds such as butanal, pentanal, hexanal, and other volatile and aliphatic compounds.[205,218]

Chemical Composition of Aloe gel

Aloe gel, the colourless mucilaginous gel contained in the parenchyma cells of the inner part of fresh Aloe leaves, is reported to contain over 200 chemical constituents, and considered to attribute to majority of the beneficial effects of the plant.[214] The gel is made up mainly of water (98.5% to 99.5%) and has a pH of 4-5.[216,220] On a dry matter basis, the gel consists primarily of carbohydrates (60%), but also contains several

other classes of compounds such as proteins (6%), lipids (4%), and other compounds such as amino acids, vitamins, enzymes, inorganic, and small organic compounds[212,214,215,221,222] (Table 4).

Aloe, being a crassulacean acid metabolism plant, survives water stress by synthesising monosaccharides and water-retaining polysaccharides, which are also responsible for the succulent nature of the plant.[223] The monosaccharides comprise 20-30% of the total dry matter of the Aloe gel,[224] reported to include galactose, arabinose, rhamnose, aldopentose, fucose and xylose,[225,226] with the most predominant monosaccharides being mannose and glucose[224] (**Paper V**). The remaining carbohydrate percentage (30-40%), also the primary component of the Aloe dry matter is made up of polysaccharides, which have been attributed to various therapeutic properties.[209,212,214] Acemannan, a mannose-containing acetylated polysaccharide is structurally unique compared to other plant mannans and reported as the majority polysaccharide in Aloe gel.[221] Acemannan is a β-(1, 4)-acetylated polymannan composed of repeating units of mannose (93%), glucose (3%) and galactose (3%), that are acetylated at the C-2 and C-3 positions,[212,221,227] with various chain lengths and molecular weight in the range of 3000Da to 5000KDa. Although acemannan is the most widely studied Aloe polysaccharide and considered as its main active substance, (biologically active substances are discussed further under the section *Bioactive composition of Aloe),* several other polysaccharides such as galactan, arabinan and glucomannan have also been detected in Aloe gel.[212,214]

Apart from the primary carbohydrate portion, the Aloe gel has also been described to contain glycoproteins, various amino acids including arginine, asparagine, glutamate, aspartate and serine, as well as at least six enzymes such as bradykinase, cellulase, carboxypeptidase, catalase, amylase, and oxidase.[228] The presence of several vitamins with antioxidant capacity, including vitamin A, C, E, B1, B2, B12, niacin, folic acid, and mineral constituents such as calcium, potassium, magnesium, sodium, and phosphorous have also been identified in the Aloe gel.[214]

***Table 4**. Summary of the chemical composition of Aloe*[205,214,218,221,222,229]

	Class	Compounds
ALOE LATEX	Anthraquinones	Aloin A and B (or collectively known as aloin or barbaloin), aloesin, aloeresin A, aloe-emodin, aloetic-acid, anthranol, isobarbaloin, emodin, ester of cinnamic acid.
ALOE GEL	Carbohydrates	
	Monosaccharides	Arabinose, fructose, fucose, galactose, glucose lactose, maltose, mannose, rhamnose, sucrose, and xylose.
	Polysaccharides	Acetylated mannose (acemannan), glucomannan, acetylated glucomannan, galactogalacturan, glucogalactomannan, galactoglucoarabinomannan, pectic substance.
	Nitrogen fraction	
	Amino Acid	Alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, serine, tyrosine, and valine.
	Glycoproteins	Lectins, lectin-like substance.
	Enzymes	Alkaline phosphatase, amylase, carboxypeptidase, catalase, cellulase, cyclooxidase, cyclooxygenase, lipase, oxidase, peroxidase, phosphoenolpyruvate carboxylase and superoxide dismutase.
	Minerals and electrolytes	Calcium, chlorine, chromium, copper, iron, magnesium, manganese, potassium, phosphorous, sodium, zinc.
	Vitamins	Ascorbic acid (vitamin C), carotenoids, thiamine (vitamin B1), riboflavin (vitamin B2), niacin (vitamin B3), B6, folic acid (vitamin B9), tocopherols (vitamin E), and choline.
	Organic acids	Acetic acid, citric acid, formic acid, fumaric acid, lactic acid, malic acid, pyruvate, salicylic acid, succinic acid and tartaric acid.
	Phytosterols	β-sitosterol, campesterol.
	Miscellaneous trace compounds	Volatile compounds: acids, aldehydes, ketones.

Further, other minority components, that may be biologically active, including phenolic compounds and organic acids, among which malic acid and citric acid, have been emphasised in Aloe gel. Lastly, fatty acids, sterols like β-sitosterol, and salicylic acid have also been detected in the Aloe gel.[205,216] Aloe extracts used in this thesis are inner leaf gel extracts, containing satisfactory amount of acemannan,[220,230] i.e. at least 5% dry weight content and safe limit of anthraquinone,[231,232] (generally considered safe at ≤10ppm aloin) for Aloe products intended for oral consumption in humans.[230] They also contain glucose, fructose, malic acid, and citric acid (**Paper II – V**).

Aloe gel has been subjected to several scientific studies aimed to characterise the carbohydrate rich composition of Aloe gel[221,233] and the phenolic composition of the anthraquinone rich Aloe latex or yellow exudate.[212,218] There are however several discrepancies in the composition of Aloe, which may be attributed to several factors including geographical location, seasonal changes and most importantly processing and extraction methods[212,233,234] (**Paper V**).

Aloe commercial products and their quality control

Although the Aloe leaf with its various parts and chemical components described above is the starting material of all commercially available Aloe products, the different extraction and processing methods have a major impact on the final product. The general production process of Aloe products involves crushing, griding or pressing of the leaf followed by various filtration, sterilisation, and dehydration steps.[211,214]

There are generally three main commercially available Aloe products depending on the part of the leaf used. First, the whole leaf product that is manufactured by grinding the entire Aloe leaf, followed by sterilisation, filtration, decolourisation using activated charcoal to remove impurities as well as majority anthraquinones. Second, the inner leaf gel product that is manufactured in a similar way to the whole leaf product, except the outer rind is manually or mechanically removed, prior to the pressing and filtration steps. Third, the Aloe latex that is extracted from the pericyclic vascular bundles.[203,214] Aloe products are offered as gels, liquids, or more often commercialised as powdered concentrate, dehydrated using various methods including spray-drying and freeze-drying, to give a dry concentrated extract.[215]

Aloe gel extracts studied in this thesis were 200:1 freeze-dried decolourised inner leaf Aloe gel extracts **(Paper II – V)** (Box 1). The extracts included in **Paper II – IV** contained less than 0.1ppm of anthraquinones and various extracts with aloin content ranging from 0.1ppm up to 10ppm were evaluated in **Paper V**. All extracts contained satisfactory amount of acemannan,[220,230] i.e. at least 5% dry weight **(Paper II – V)**.

BOX 1. DESCRIPTION OF STUDY ALOE EXRACTS

200:1	Concentrated solids relative to fresh Aloe inner leaf gel
Freeze dried	Dehydration method used to convert liquid gel into a powdered form
Decolourised	A purification process including filtration with activated charcoal to remove unwanted phenolic compounds (anthraquinones including aloin)
Inner leaf gel	Describing the part of Aloe leaf used as starting material for extract production i.e., the central parenchymatous tissues of the Aloe leaf

The different manufacturing processes as well as the different liquids and extracts produced from Aloe leaves are often referred to with interchangeable terms, which has led to inconsistencies in the scientific as well as the lay literature. The commonly used terms in the Aloe industry are described in Table 5. In accordance with the pharmacopeial definitions,[203,208,217] the term “Aloe gel” should be used for the inner mucilaginous clear gel extracted from the leaf parenchyma tissue and the “Aloe juice” should be restricted to the yellow exudate or Aloe latex extracted from the pericyclic tubules.

Although Aloe has a long history of use as a herbal remedy, the quality control of Aloe or Aloe preparations have not been extensively studied.[220] Recent evidence, suggesting the carcinogenic nature of Aloe whole leaf extract[213,231,235] (discussed in section *Toxicological*

properties) as well as the continuous adulteration of Aloe products owing to the high raw material cost, have generated interest in controlling the authenticity and safety of Aloe products. In this regards, acetylated polysaccharides (acemannan) and malic acid (components found exclusively in the Aloe gel), and aloin (found predominantly in the Aloe latex) are some Aloe components which have been identified as the quality and safety markers of Aloe.[236,237]

***Table 5**. Definitions of terms commonly used in the Aloe industry[238,239]*

Term	Definition
Aloe leaf	The part of the Aloe plant used for manufacturing commercially available products.
Aloe gel	Clear gel product typically derived from the inner leaf that contains Aloe pulp.
Aloe latex	Brown, yellow-brown, or occasionally red exudate found sandwiched between the rind and inner leaf. Also known as the "Aloe sap" or "Aloe juice". It notably contains anthraquinones such as aloin. When dried, it has been used as a bittering agent in foods and beverages, and as a laxative.
Aloe juice	Used to characterise Aloe latex in pharmacopeias.[217] However also confused and used in general to describe liquid products derived from Aloe leaf.
Anthraquinones	An organic compound primarily found in the Aloe latex and the substance commonly utilised for laxative purposes. Example: aloin.
Aloin	The major anthraquinone glycoside compound found in Aloe latex.
Decolourisation	A purification process including filtration with activated charcoal to remove unwanted phenolic compounds (anthraquinones, including aloin). Both whole leaf products and inner leaf gel products can be purified using decolourisation.
Dehydration	The method used to convert liquid gel into a powdered form that can be carried out using various methods including spray-drying and freeze-drying.
Inner leaf product	Describing the part of Aloe leaf used as starting material for production i.e., the central parenchymatous tissues of the aloe leaf.
Whole leaf product	Refers to the starting ingredient i.e., the entire leaf used to create Aloe product.

Currently, the only organisation which sets the industrial standards for commercially available Aloe products is 'The International Aloe Science Council' (IASC). This is a non-profit trade organisation for the Aloe industry worldwide which carries out certification of good quality Aloe products in accordance with the general food safety requirements set by authorising bodies of each country and based on standards set by the IASC (Table 6). These requirements are generally guided by three fundamental aims: confirming authenticity, recognition of adulteration and controlling the aloin content.[230,238,239] Keeping in mind the potential carcinogenic properties of Aloe latex at high doses,[231,240,241] a maximum allowed concentration in all commercial Aloe products has been set by the IASC and the Cosmetic Ingredient Review Expert Panel (Box 2). While these guidelines and certification process enable authenticity and general safety of Aloe products, it should indeed be kept in mind that the chemical composition and quality standards of commercially available Aloe products vary greatly depending on the starting material, different geographic location, collection season, production processing, and storage[220,237] (**Paper V**).

***Table 6**. IASC quality standards for commercial Aloe products intended for oral consumption[220,230]*

Component	**Origin of Component**	**IASC Aloe Standard** Content by dry matter
Acemannan	Fresh Aloe Vera	≥5%
Malic acid	Fresh Aloe Vera	Present
Glucose	Fresh Aloe Vera	Present
Aloin	Anthraquinone	≤10ppm
WLM (Isocitrate)	Whole leaf marker	≤5%
Maltodextrin	Adulterant/Additive	Absent if undeclared

NOTE: Content % data refers to dry matter

Box 2. **ALOIN CONTENT IN COMMERCIAL ALOE PRODUCTS[230,242]**	
Products for oral consumption	<10ppm
Products for topical usage	<50ppm

Toxicological properties

Aloe has a long history of topical use, but also oral ingestion including the traditional use of Aloe latex or "Aloe juice" rich in anthraquinones for the short-term treatment of occasional constipation, which is well described in several monographs and pharmacopoeias.[203,208,217] Despite its widespread usage, recent studies have raised concerns regarding the safety of orally administered Aloe.[213,240,241,243] These studies, including a report issued by the National Toxicology Program of the United States and studies by Boudreau *et. al.*[213,235,241], focused on whole leaf Aloe extract. The report demonstrated increased incidences of adenomas and carcinomas of rat caecum and large intestine and provided evidence implicating non-decolourised, high-anthraquinone containing Aloe preparations with carcinogenic effects.[213,240,241,243] Owing to the insufficient data to establish the safety of Aloe latex for use as a laxative, it is no longer recognised as an over-the-counter drug by the U.S. Food and Drug Administration.[244] Recently it has also led to new regulatory measures by the European commission, prohibiting the use of Aloe hydroxyanthracene derivatives in foods and food supplements, setting the level of 1ppm for aloe-emodin/emodin and the sum of aloins A and B as the threshold in food products.[245]

It should however be pointed out that the Aloe extracts used in the toxicology studies mentioned above contain a total aloin content more than 1000 times higher than the industry standard set by the IASC,[231] as described in the previous section. These non-decolourised, high-anthraquinone Aloe extracts are not representative of the marketplace standard Aloe products for oral consumption,[231] where majority of phenolic compounds existing in the latex or contaminated from the latex are usually removed through activated charcoal filtration. Furthermore, studies assessing the safety of orally administered decolourised Aloe gel preparation (with total aloin content ranging from 0.1ppm up to 10ppm), similar in description to the extracts used in the studies included in this thesis (**Paper II – V**), were well-tolerated and reported absence of genotoxicity[246] and no toxicologically significant findings for oral consumption of up to 3 years.[243,247-249] These results are consistent with several other studies involving charcoal-processed, freeze-dried Aloe extracts,[250,251] which although do not report the total anthraquinone concentration of the preparations used, still support the safety of Aloe gel and gel products containing limited aloin.

ALOE BARDADENSIS MILL. AS A THERAPEUTIC OPTION FOR PATIENTS WITH IBS

Aloe barbadensis Mill. (Aloe) with its historic reputation as an herbal medicine has been used for centuries in several cultures for its diverse therapeutic applications.[203-205] Although traditionally known for its topical use,[249] the therapeutic claims for the oral consumption of Aloe are wide-ranging,[205,252-254] as are the pharmacological activities associated with it[216,255-257] (discussed further under section *Therapeutic properties of Aloe - potential mode of action in IBS*). The diverse therapeutic properties of Aloe, together with the limitations in the currently available treatment options for the complex and multifactorial IBS, has made Aloe an attractive alternate therapeutic strategy for alleviating abdominal symptoms and discomfort in patients with IBS.

Despite its widespread use and a large percentage of IBS patients stating the successful use of Aloe products to manage their symptoms,[258-260] there is a paucity of controlled clinical trials and only a few previous studies have evaluated Aloe as a therapeutic option for patients with IBS.[258,259,261,262] Therefore, we evaluated the effects of treatment with Aloe gel derived extract on symptoms in patients with IBS in a randomised, double-blind, controlled study described in **Paper II,** where the study size was determined using power calculation based on a pilot study performed by our group[259] (Figure 7).

In consensus with previous clinical studies and the general safety of Aloe gel products (discussed under section *Toxicological properties*), treatment with Aloe gel extract had no adverse effects and was well tolerated and safe for patients with IBS (**Paper II**). Supporting the conclusion from a recent meta-analysis,[263] our study depicted beneficial treatment potential of Aloe extract, at least in subsets of IBS patients, with more than a third of the patients in the Aloe group responding to the treatment (Figure 8). In this study, response was defined as reduction in IBS Severity Scoring System (IBS-SSS) $\geqslant$ 50 points at the end of the treatment period relative to baseline.[69] However, echoing previous studies,[258,259,261] our study failed to demonstrate superiority of Aloe treatment to control treatment in alleviating overall symptom severity in the mixed group of IBS patients including all IBS subtypes

(**Paper II**). This is not surprising and could potentially be owed to the heterogenous underlying pathophysiology of IBS, which may also be the main culprit for the limited benefit over placebo and other control treatments obtained in several other IBS therapeutic trials.[54] Nevertheless, the improvement seen in the bowel habit dissatisfaction score, exclusively by the Aloe treatment (**Paper II**), was in line with the previously reported beneficial effects of Aloe in improving individual IBS symptom scores such as IBS pain score including both pain severity and frequency of pain, as well as improving abdominal bloating.[259,262] The existing data in the literature is however inconsistent and should be interpreted with caution, where one study was unable to demonstrate any beneficial effects of Aloe,[261] and others reported improvement of symptoms only in the diarrhoea predominant[258] or the constipation predominated[262] subtypes of IBS patients. A prevalent limitation and reason for the varying results could indeed be due to the inconsistent form, part or dose of Aloe used as the active treatment in the different clinical studies.[258,259,261,262] The '*Bioactive composition of Aloe*' as an important consideration for its therapeutic properties will be discussed in the following sections.

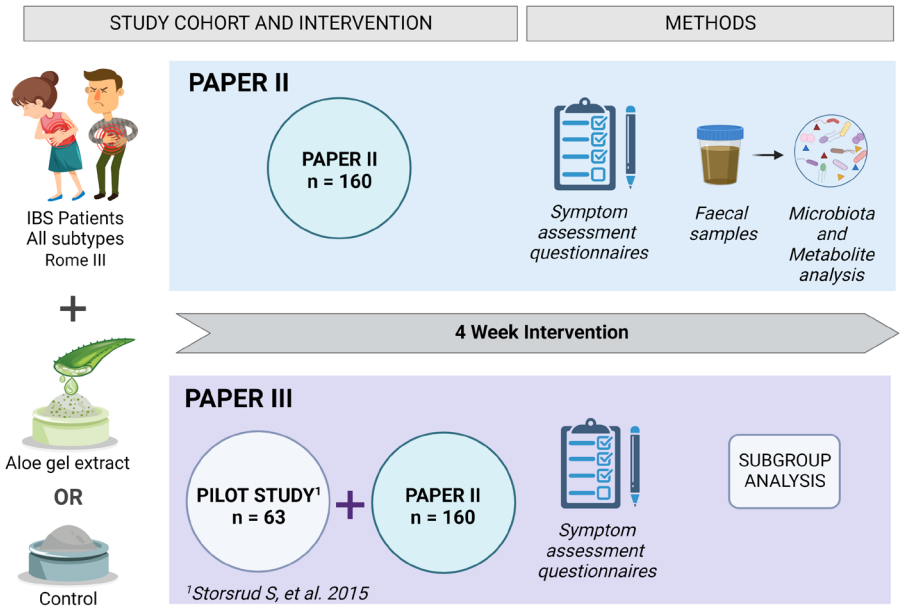

Figure 7. *Overview of the study design for the controlled trials evaluating the therapeutic effect of Aloe barbadensis Mill. (Aloe) in patients with IBS and IBS subgroups. Created with BioRender.com.*

Considering the heterogeneity of IBS, subgrouping of IBS patients is a common clinical practice when recommending treatment for the management of IBS symptoms.[54] The therapeutic efficacy of Aloe in various patient subgroups of IBS has however not been sufficiently evaluated in clinical studies. While our study, in agreement with a previous clinical study,[258] depicted a tendency of improved overall symptom severity in the diarrhoea predominant IBS patients (IBS-D) (**Paper II)**, the study was underpowered and hence conclusions regarding Aloe treatment effects in IBS subgroups could not be established. To address this, in **Paper III** we therefore conducted a post hoc analysis of pooled data from the two randomised controlled studies by our group (Pilot study[259] and **Paper II)** to evaluate the treatment effect of Aloe extract on IBS symptoms in the various IBS patient subgroups, based on predominant bowel habit, IBS symptom severity, as well as psychological distress (Figure 7). Our results demonstrated that Aloe extract had a significant effect in alleviating overall symptom severity in IBS-D patients, with pronounced improvement in abdominal pain severity and pain frequency (**Paper III)**. This beneficial effect was also reflected as clinically meaningful improvement with a higher frequency of responders (defined as reduction in IBS Severity Scoring System (IBS-SSS) $\geqslant$ 50 points at the end of the treatment period relative to baseline[69]) in response to Aloe compared to the control treatment in the IBS-D group (**Paper III)** (Figure 8). Further, Aloe treatment showed a tendency towards improved overall symptom severity in a small group of IBS patients with depression, but it was not superior in alleviating IBS symptoms over control treatment in other patient subgroups based on symptom severity or psychological distress (**Paper III)**.

Altogether, the current available data, supports that treatment with Aloe gel extract is safe and well tolerated by patients with IBS and can be regarded as a promising treatment option in the management of IBS symptoms, particularly in subsets of IBS patients (**Paper II and III**) (Figure 8).

To improve the efficacy of Aloe gel as a therapeutic option for IBS patients, keeping in mind the complex and multifactorial nature of the disease, there is however a need to understand better how Aloe treatment works, as well as to identify biomarkers that may help predict the treatment response. In this regard, faecal microbiota and metabolite composition evaluated as secondary endpoints in **Paper II,** depicted that IBS patients responding to Aloe treatment displayed distinct faecal microbiota and metabolite profiles compared to non-

responders before the treatment. Hence, the treatment outcome to Aloe in IBS patients, including all subtypes, could be predicted by gut microbiota-metabolite composition. A favourable treatment outcome was associated with higher abundance of *Akkermansia muciniphila,* an intestinal microbe associated with a healthy gut[264] and alterations in various metabolites related to monosaccharides as well as amino acid metabolism before the start of the intervention. Our findings are suggestive of differences in the gut microenvironment between the Aloe treatment responders and non-responders (**Paper II**). Although warranting confirmation, gut microenvironment, composed of the gut microbiota and metabolites, may be a predictive biomarker to help identify IBS patients likely to benefit from Aloe treatment for improvement of symptoms.

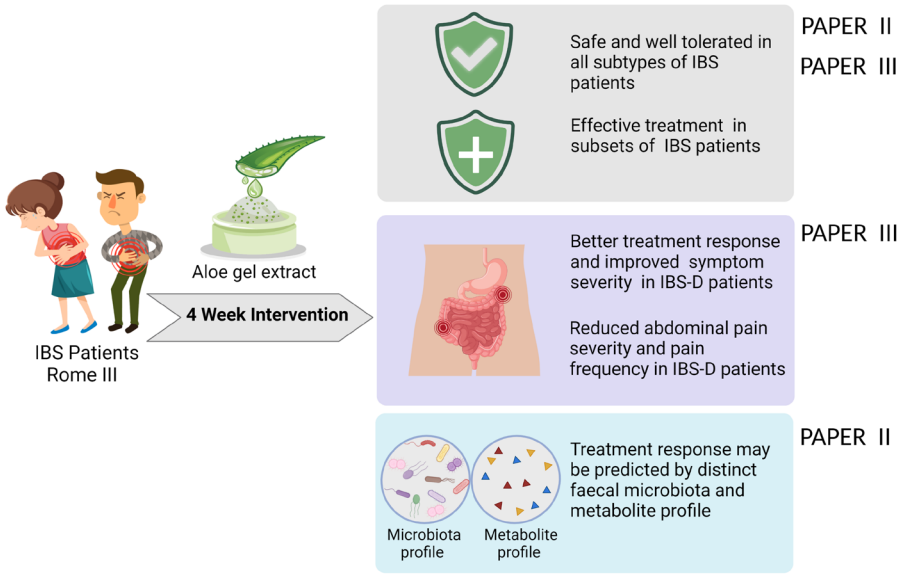

Figure 8. *Therapeutic effects of Aloe barbadensis Mill. (Aloe) in patients with IBS patients and IBS subgroups (Paper II and III). Created with BioRender.com.*

Furthermore, the elusive mode of action for the beneficial effect of Aloe supplementation in IBS patients was also investigated in **Paper II,** where the effect of Aloe treatment on faecal microbiota and metabolite profiles was evaluated. This was also addressed in **Paper IV**, which focused on the effect of Aloe gel extract on immune cell activity. Considering the relevant role of an altered intestinal microenvironment and mucosal immune function in the pathogenesis of IBS, the potential

immunomodulatory and prebiotic properties of Aloe are potential modes of action for the therapeutic effects of Aloe in IBS patients and are discussed in the section below.

THERAPEUTIC PROPERTIES OF ALOE - POTENTIAL MODE OF ACTION IN IBS PATIENTS

The reported health benefits of Aloe gel are numerous and have been attributed to its well-known and wide-ranging therapeutic properties[205] (Figure 9). The promising effects of Aloe gel in treatment of digestive disorders, mild to moderate ulcerative colitis and peptic ulcers[205,254] have been associated with its wound healing, antioxidant, antimicrobial, anti-inflammatory, as well as immunomodulatory properties.[205,254-256,265-267] The decrease in gastric acid secretion and increase in mucus secretion, reported as effects of Aloe gel preparation,[268,269] further favours its therapeutic role in GI disorders. While the mode of action for the beneficial effect of Aloe treatment in IBS remains elusive, it could indeed be credited to its diverse therapeutic properties.

Considering the relevant role of an altered mucosal immune function and low-grade inflammation in the pathogenesis of IBS,[76] characterised by changes in both the innate and adaptive immune system, including increased immune cell counts, particularly T cells as well as elevated levels of proinflammatory cytokines,[74-76,86] the anti-inflammatory properties of Aloe could be of particular interest. Thus, our study for the first time investigated the effects of Aloe gel extract on human blood T cell activity *in vitro* using polyclonally stimulated peripheral blood mononuclear cells from healthy donors (**Paper IV**) (Figure 10). In agreement with several previous studies,[254-256,265,270,271] our results demonstrated immunosuppressive effects of Aloe gel extract, defined by reduced T cell activation and proliferation (depicted as reduced CD25 expression among polyclonally activated T cells and reduced CFSE and thymidine assessed proliferation, respectively), as well as reduced T cell secretion of cytokines IL-2, IFN-γ and IL-17A (**Paper IV**). Further, our study suggested the reduced expression intensity of CD28, a co-stimulatory molecule known to play a role in the complex cascade of reactions involved in T cell activation and proliferation, seen among $CD3^+$ T cells, as a possible mechanism for the reduced T cell proliferation caused by Aloe gel extract. Although adding to the general

understanding and mechanism of action of Aloe gel, the exact mode of action for the activity of Aloe gel on T cells remains unclear.

The extrapolation of results from **Paper IV** into a clinical setting is difficult and more studies are warranted. Still, the suppressive effects of Aloe gel *in vitro*, shown to be comparable to the well-established immunosuppressive drug Infliximab, without causing cell apoptosis or cell death (**Paper IV**), supports the safe use of Aloe derived gel extract as a therapeutic option for GI diseases.

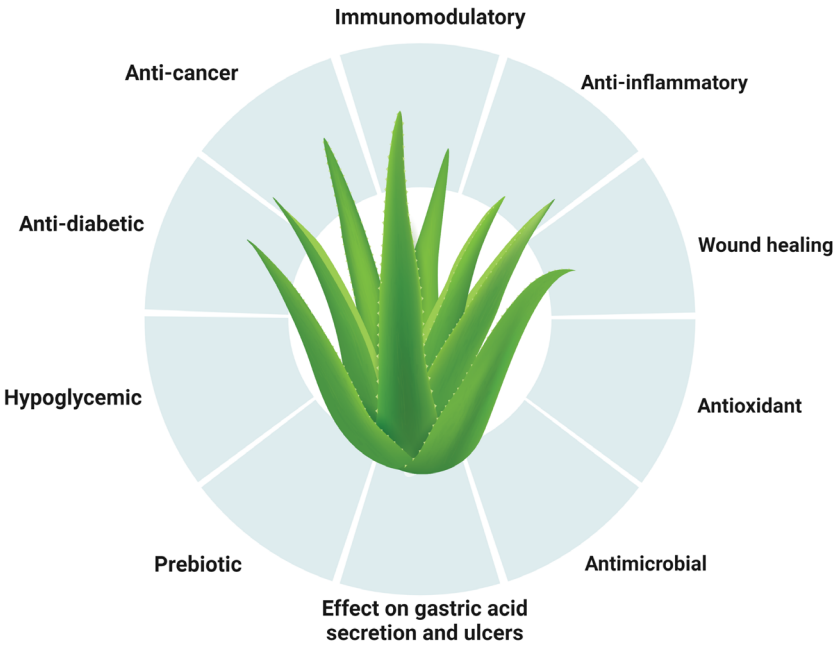

Figure 9. *Wide ranging therapeutic properties associated with Aloe gel. Created with BioRender.com.*

Apart from the role of an altered immune function, mounting evidence claims the importance of an altered intestinal microenvironment in the pathogenesis and pathophysiology of IBS.[31,98,99] Further extending the diverse therapeutic properties of the polysaccharide rich Aloe gel, it has recently been shown to possess prebiotic properties, as the ability of Aloe gel to promote growth of beneficial colonic bacterial populations was associated with possible improvement in GI health.[272-274] While some studies indicate the possibility of using Aloe extracts as prebiotics,[272,274] no previous studies have investigated the effect of Aloe

treatment on the intestinal microenvironment in IBS patients. Thus, in **Paper II,** evaluating faecal microbiota and metabolite profiling of IBS patients treated with Aloe extract, we for the first time, suggested potential mechanistic relationships between Aloe treatment response and gut microbiota-metabolite composition in patients with IBS. In view of the relative stability of the gut microbiota composition, seen during severe intestinal inflammation,[37,275] our study did not depict any major change on the overall gut microbiota and metabolite profiles associated with the treatment groups. Nevertheless, the faecal microbiota and metabolite profiles of responders to the Aloe treatment differed from that of the non-responders before the treatment period, indicative of its predictive ability (as described in the section above). Moreover, the faecal microbiota and metabolite profile of responders also differed from that of the non-responders after the treatment period, described as change in the abundance of several bacterial genera, including increased abundance in *Bacteroides,* gut bacterial taxa associated with health, as well as change in concentration of several different metabolites (**Paper II**). Hence, our study suggests differences in the gut microenvironment of responders to the Aloe treatment as compared to the non-responders. This difference may be due to the ability of their gut microbiota to utilise the components of the Aloe treatment, leading to a shift between protein and carbohydrate metabolism, hence indicative of a functional change in their gut microenvironment (**Paper II**).

While the mode of action for the beneficial effect of Aloe treatment seen in subsets of IBS patients (**Paper II** and **III**) remains far from completely understood, it could undeniably be a collaborative effort of multiple mechanisms. Considering the complex and multifactorial pathophysiology of IBS, treatment with Aloe gel extract attributed with a battery of therapeutic properties may indeed be considered advantageous over pharmacological agents that have more precise modes of action and are often accompanied by risk of adverse effects.

BIOACTIVE COMPOSITION OF ALOE

Aloe gel, the colourless mucilaginous gel from inner parenchymatous Aloe leaf, attributed with diverse therapeutic benefits,[234,255-257,265,266,276,277] including alleviating abdominal symptoms in subsets of patients with IBS (**Paper II and III**) and suppressive effects on immune cell activity (**Paper IV and V**), is one of the most popular medicinal plants used worldwide. Despite its extensive use and several scientific studies over the last decades, there is still no clear correlation between the beneficial properties of Aloe gel and its composition, and the debate regarding the component or group of components accountable for the various therapeutic properties remains unsettled.

Aloe gel contains a rich source of numerous biologically active or "bioactive" components. Amongst these, its complex polysaccharide composition, in particular acetylated mannose, also called acemannan, besides its use as a quality marker for commercial Aloe products, has also been implicated with several therapeutic properties and considered to hold the secret for the beneficial effects of Aloe gel.[233,278-280] Other chemical components which have been implicated for the various therapeutic effects of Aloe gel include mannose-6-phsphate,[265] sterols,[281] β-sitosterol,[282] and glycoproteins.[283,284] Additionally, recent studies have also correlated beneficial properties of Aloe to its high-molecular weight polysaccharide fractions.[285]

While some studies have focused on isolating individual bioactive component(s) or fraction(s) from Aloe gel, responsible for its therapeutic effects, it is believed that the beneficial properties of Aloe gel should be assigned to a synergistic action of several compounds rather than a single chemical compound.[205,209] In line with this, the studies supporting the promising immunosuppressive effect of Aloe[256,257,286-295] vary in the form or composition of Aloe gel used. Additionally, results from **Paper IV** and **Paper V** comparing the effect of various Aloe gel extracts on T cell activity, demonstrated that extracts with distinct chemical compositions differed in potency of their immunosuppressive effect (Figure 10). Further, in contrast to its anti-inflammatory activity, Aloe gel and its components such as acemannan have also been implicated to have immunomodulatory, immunostimulatory or even undesirable properties.[212,296-299] These divergent results make it presumptuous to correlate the immunosuppressive effect of Aloe with only one specific chemical

compound and further emphasises the complex and synergistic nature of Aloe gel.

Adding to the complexity is the quantitative and qualitative variation in the bioactive composition of Aloe gel products (liquid and dehydrated extracts), which may be influenced by several factors, including horticulture practices and most importantly processing and extraction methods.[212,233,234] Commercial Aloe products (described in the section *Aloe commercial products and their quality control)* are usually offered as gels, liquids, or dehydrated products and they vary greatly in their starting material and method of processing. Hence, the final products are likely to have very different chemical/bioactive composition.

Thus, we need to improve our understanding of the complex bioactive composition of Aloe. In **Paper V**, along with comparing the effect of various commercially available Aloe gel extracts on human blood T cell activity *in vitro* and correlating this effect to their standard phytochemical quality characteristics, we also for the first time compared and correlated the effect of Aloe gel extracts on immune cell activity through metabolomics (Figure 10). Although metabolomics, the profiling of a wide range of small molecules in a sample, has been exploited to characterise the metabolite composition of various plants,[300,301] knowledge regarding the metabolite composition of Aloe gel remains sparce.

Results from our study demonstrated that despite similar commercial description, the Aloe gel extracts varied in their standard phytochemical quality characteristics, as well as in their effect on human blood T cell activity *in vitro* (**Paper V**). Contradicting common belief, acemannan which is often linked to the potency of Aloe, was not the determining factor for the varied response seen on stimulated T cells in our *in vitro* study. Further, the effect could not be attributed explicitly to the high-molecular Aloe polysaccharide composition (**Paper V**). The novel results from our study demonstrate that each Aloe extract was unique with its own distinct metabolite profile, where a profile rich in sugars and sugar derivates such as 2-deoxy-glucose, n-acetylglucosamine and glucosamine, could be correlated to its effects on T cell activity and proliferation *in vitro* (**Paper V**).

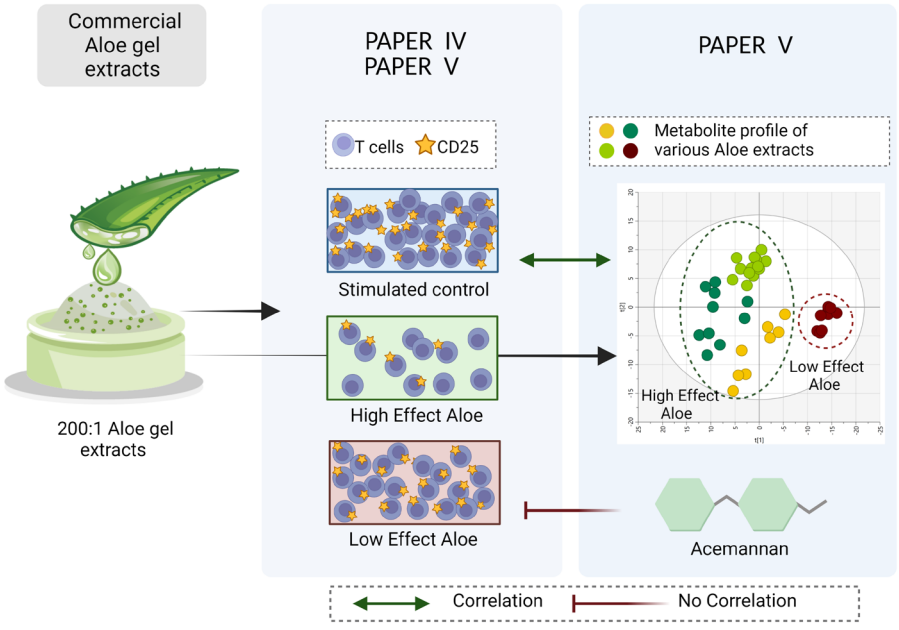

Figure 10. Effect of various commercial Aloe gel extracts on immune cell activity in vitro and correlation of the effect to their chemical composition (Paper IV and V). *Aloe gel extracts with similar commercial description (200:1) vary in their effect on human blood T cell activity. Further, all Aloe extracts are unique with distinct metabolite profiles, which lead to differential immune activity in vitro, independent of the acemannan content. Created with BioRender.com.*

More work is indeed needed to understand the importance of extraction and processing parameters for Aloe extracts, which imprint substantial differences in their metabolite composition, making them unique. Future focus should be directed towards exploiting metabolomics to add on to the existing phytochemical profile of Aloe, to enable further the design and formulation of future functional foods and therapeutic products. Overall, our results advance our understanding of the complex bioactive composition of Aloe gel, which is indeed synergistic in action. We suggest that it is not possible to assume that all Aloe extracts are similar and while each Aloe extract with its unique metabolite profile may possess beneficial properties, important consideration should be given to identifying and linking bioactive composition of Aloe extracts with the desired biological effects.

METHODOLOGICAL CONSIDERATIONS AND LIMITATIONS

In this thesis, clinical data and faecal samples (Table 7), collected from controlled studies, were evaluated to assess the importance of intestinal microenvironment in IBS and determine the therapeutic effect of *Aloe barbadensis* Mill. (Aloe) gel extract intervention in patients with IBS (**Paper I to III**).

Table 7. *Overview of study participants and assessments in Papers I to III.*

	Paper I	**Paper II**	**Paper III**
Study participants	40 IBS patients (Rome III) [from Paper II] and 18 Healthy subjects[105]	160 IBS patients (Rome III) (84 Aloe group) (76 Control group)	63 IBS patients (Rome III) – [from Pilot study[259]] and 160 IBS patients (Rome III) [from Paper II]
Symptom assessment (IBS patients)	IBS Severity Scoring System (IBS-SSS) Bristol stool form (BSF) scale Hospital Anxiety and Depression (HAD) scale		
Faecal microbiota profile assessment	GA-map™	GA-map™	-
Faecal metabolite profile assessment	Untargeted GC-MS/MS	Untargeted GC-MS/MS	-

Further, the effect of Aloe gel extract on immune cell activity, as a potential underlying mechanism of therapeutic effect and characterisation of the bioactive composition of Aloe gel extract responsible for this effect was carried out *in vitro* (Table 8), using peripheral blood mononuclear cells (PBMC) from healthy donors (**Paper IV and V**).

Table 8. *Overview of study material and assessment in Papers IV and V.*

	Paper IV	**Paper V**
Study material (Venous blood)	18 Healthy donors	7 Healthy donors
Assessment of immune cells	PBMC; Flow cytometry	
Aloe phytochemical quality assessment	-	Targeted ^{1}H-NMR spectroscopy HPLC-UV
Aloe metabolite profile assessment	-	Untargeted GC-MS/MS

STUDY PARTICIPANTS, STUDY DESIGN AND SYMPTOM ASSESSMENT QUESTIONNAIRES

All studies included in this thesis were conducted in accordance with the declaration of Helsinki and study protocols were approved by the Regional Ethical Review Board in Gothenburg. Study participants were recruited from patients referred to the gastroenterology outpatient clinic at the Sahlgrenska University Hospital, Gothenburg, as well as through advertisement in local newspapers. All study participants received oral and written information about the study procedures and informed written consent was obtained from them before study start.

The controlled clinical studies (**Paper I – III** and Pilot study[259]) included both women and men of all subtypes of IBS, and with a broad range of symptom severity (mild to severe) and psychological distress, ensuring a representative group of patients. Thus, the findings from our studies should be transferrable to patients with IBS in general.

To study the therapeutic effect of *Aloe barbadensis* Mill. (Aloe) gel extract intervention in patients with IBS, study participants (**Paper II** and **III** and Pilot study[259]) were randomised to receive Aloe treatment (500mg Aloe and 780mg inulin/day) or control treatment tablets (1280mg inulin/day), respectively for four weeks. With both the Aloe treatment and control treatments containing different doses of inulin, a fermentable fibre with suggested prebiotic properties, the formulation of the treatment tablets in our studies is a weakness and may have led to our study (**Paper II**) not fulfilling its primary endpoint. Further, the

treatment period limited to four weeks may also be considered as a limitation in our studies.

With no well-defined indicative biomarkers, assessment of symptoms in IBS patients was carried out using several validated questionnaires. The main outcome variable in **Paper II** and **III** was IBS symptom severity, which was assessed with IBS-SSS,[69] completed on a weekly basis. BSF scale,[56] completed daily, was used to log bowel movements and to define the IBS subtypes (**Paper I to III**). Finally, psychological symptom severity was assessed using the HAD scale,[70] completed before and after the 4-week intervention period (**Paper I** to **III**). While all these questionnaires are valid and reliable, and widely used in clinical and research settings, self-reported assessment measures are at risk of biases, such as recall bias. Thus, a systematic error caused by difficulties in accurately reporting past events might have influenced our results. Another limitation in our studies was the lack of dietary habit assessment, which can affect both the gut microbiota and metabolite composition, evaluated in our studies as primary and secondary outcomes (discussed in following sections *Gut microbiota composition* and *Metabolite profiles*).

A reduction of ≥50 in the IBS-SSS score was used as cut-off to define response to treatment, as it is considered to be a clinically relevant reduction in symptom severity.[69] The IBS cohort included in our studies (**Paper I – III)** had in general relatively modest symptom severity (IBS-SSS ± SD) at inclusion (**Paper I**: 216 ± 73; **Paper II**; 283 ± 95 and **Paper III;** 293 ± 88 (Pilot study) and 283 ± 95 (**Paper II**)). This might have affected our results in **Paper II**, possibly leading to the low overall response rate seen in our study, and failure to show superiority of Aloe treatment in the mixed group of IBS patients including all IBS subtypes. However, the heterogenous IBS cohort with milder symptoms, being more representative of the average IBS patient, adds strength to our results, especially in **Paper I**, where a distinct intestinal microenvironment was depicted in IBS patients (including all subtypes).

Faecal samples, preferred over intestinal biopsies due to the ease of collection, were analysed for microbiota and metabolite composition as a proxy for the intestinal microenvironment in this thesis (**Paper I and II**). Samples were collected at baseline and at the end of treatment period, which enabled association between the treatment response and gut microbiota and metabolite profiles (**Paper II**). However, results

from **Paper I** could have benefitted from additional longitudinal sampling.

GUT MICROBIOTA COMPOSITION

Mounting evidence supports the role of an altered gut microbiota in the pathogenesis of IBS.[98,99] The commercially available GA-map™ dysbiosis test (provided by Genetic Analysis AS, Oslo) was used to depict the composition of faecal gut microbiota in our studies (**Paper I - III**). GA-map™ is a targeted DNA-based method that detects differences in the V3–V9 regions of the 16S rRNA gene for taxonomic identification of microbial communities. The analysis provides information about the absolute abundance of the targeted bacteria, based on 54 DNA probes targeting ≥300 bacteria of different taxonomic levels and generates a bacterial profile based on 15 different bacteria (defined by Genetic Analysis AS), thus, providing an overview of specific bacterial taxa that are known to be associated with GI conditions.[302]

The easy-to-use GA-map™ analysis has the benefit of being reproducible, fast, less expensive, straight forward, easy to interpret and provides absolute abundance of targeted bacteria,[302] it however has limitations when compared to the more elaborate untargeted 16S rRNA gene sequencing. The untargeted gene sequencing analysis uses the V3-V4 region and provides the relative abundance of bacterial taxa present in the sample.[303] This high-throughput gene analysis gives a detailed picture of the microbial community in the analysed samples and has the added advantage of providing information about species richness and diversity.[302,303] However, being more expensive, time consuming and complex to analyse, are some disadvantages of this method. Further, its limited ability to provide relative abundance of microbial species up to taxonomical level, makes comparisons between different samples challenging.

Our studies (**Paper I** and **II**), being of explorative and hypotheses-generating nature, could have benefitted from the high-throughput untargeted gene sequencing analysis. Still, the detection of GI related bacterial taxa and the possibility of comparison between samples, to study changes in gut microbiota profiles potentially associated with IBS patient group compared to healthy subjects, is an advantage of our chosen method.

METABOLITE PROFILES

To understand the mechanisms by which the gut microbiota drives disease and symptom pathophysiology in IBS, metabolite composition as a functional output or a snapshot into the complex host-microbial interaction is regarded as a valuable tool.[45,127]

Gas chromatography coupled to a tandem mass spectrometer (GC-MS/MS) was the method chosen in this thesis for the analysis of metabolomic profile of faecal samples from the study subjects (**Paper I - II**) and the various Aloe extracts (**Paper V**). While GC-MS/MS is a relatively easy, cheap, and quick method, where MS enables good separation of samples and increase in sensitivity,[304] it is however restricted to small compounds that are thermally stable, volatile, or can be made volatile through derivatisation.[304,305] Liquid chromatography coupled to a mass spectrometer (LC-MS) which is now commonly used for metabolomics, was not initially available when starting this project. LC-MS has advantages over GC-MS, primarily with the breadth of metabolites that can be detected. However, most LC-MS metabolomics methods require a lot of resources to annotate a few compounds with fair certainty.[305] The primary advantage of using GC-MS in this project is the ability to annotate detected features rapidly and confidently, especially those related to central metabolic pathways. Apart from the chromatography (GC and LC) coupled-MS, ^{1}H-NMR spectroscopy, with its high reproducibility, and the ability to simultaneously quantify multiple classes of metabolites, is another technique used for metabolomic analysis.[305] Due to its relatively low sensitivity, it was not used for analysis of faecal metabolite profile of study participants in **Paper I** and **II**, or for the evaluation of metabolite profile of various commercial Aloe extracts in **Paper V**. It however formed the basis of the commercially available ^{1}H-NMR spectroscopy certification method used for the authentication and quality assessment of Aloe extracts performed at Spectral Service AG (Köln, Germany).[236,237]

More extensive in-depth metabolomic analyses could have been preferential to understand the change and differences in metabolite profiles seen between patients and healthy subjects (**Paper I – II)**, as well as between various Aloe extracts (**Paper V)**. Balancing the objectives of the overall project against depth of information, we selected GC-MS as the most appropriate method for metabolomics, due to general coverage of primary metabolic pathways and good resources for annotation of metabolic features. This has allowed us to identify

that metabolite profiles can help to describe differences between patients and healthy subjects (**Paper I** - **II**), and Aloe extracts (**Paper V**). Future work using wider metabolome coverage possible with LC-MS will help us to build upon and expand our present results.

IN VITRO MODEL TO STUDY EFFECT OF ALOE ON IMMUNE CELLS

To compare and assess the effect of various Aloe extracts on human blood T cell activity (**Paper IV** and **V**) we carried out a straightforward and repeatable *in vitro* assay using polyclonally stimulated peripheral blood mononuclear cells from healthy donors in the presence or absence of Aloe extracts. Results from our studies further added to the understanding of the effects of Aloe gel extract on immune cells and correlated the effect to their bioactive composition.

Although *in vitro* studies have several advantages including control of environment, low cost, higher throughput, and reduced animal-use, they have substantial weaknesses of being unable to replicate the conditions in an organism.[306] Thus, an extrapolation of our *in vitro* results into a clinical setting is difficult and warrants further studies. To improve and validate our results, the use of other multifaceted *in vitro* models such as co-cultured epithelial and immune cells in transwells[307,308] and exploitation of more complex models based on human derived organoids[309] could indeed be advantageous.

MULTIVARIATE DATA ANALYSIS

Throughout this thesis, several complex microbiota and metabolite datasets, comprising of a large number of variables, have been analysed to understand IBS and the therapeutic effects of Aloe (**Papers I, II** and **V**). To focus on the relevant information contained in these large complex datasets, they were evaluated simultaneously using multivariate statistical analysis.

In **Paper I** the multivariate data was analysed using an unsupervised method, Principal component analysis (PCA). PCA reduces the number of dimensions while preserving all the valid information,[310] which enables visualisation of clusters or similarities in biological samples and helps filter out noise. It helps to depict trends based on correlation between several independent variables that influence a dependant

variable, without considering underlying assumption of the variables included.[310] PCA however cannot be used for the purpose of identifying differentiating variables between two groups.

To address this, a supervised method, Orthogonal partial least square-discriminant analysis (OPLS-DA), was used for identifying discriminatory variables between groups, such as "Healthy" and "IBS", treatment "Responders" and "Non-responders" or "High Effect Aloe" and "Low Effect Aloe" in **Papers I, II** and **V**. The OPLS-DA ranks variables according to their importance in the differentiation of the selected groups and these are visualised in loading plots, where the most discriminating variables are found furthest away from the origin.[311] The supervised models however suffer from risk of overfitting, since separation is "forced" and can cause even random data to show discrimination and hence must be interpreted with caution.[312] The R^2 and Q^2 values can be used to assess the model robustness and predictive power, respectively (**Papers I, II** and **V**). Model validation approaches, such as CV-ANOVA and permutation tests (**Paper II**) can further be used to confirm model robustness, along with using validation samples and cohorts to improve bias.[312] Further confidence in the findings from multivariate statistical analysis can be gained by additionally applying univariate methods such as t-tests or correlation or more advanced univariate modelling.

CONCLUSIONS AND FUTURE PERSPECTIVES

Irritable bowel syndrome (IBS) is one of the most common and extensively investigated functional GI disorders. Despite the progress made in the last decade to understand this multifactorial disorder, its pathophysiology remains incompletely understood. The research described in this thesis strengthens the role of an altered intestinal microenvironment in the pathogenesis of IBS. Interestingly, our results support IBS to be more strongly associated with an alteration in the metabolite profile rather than in the microbial composition, highlighted by shifts in pathways involving amino acid metabolism. Nevertheless, the integrated microbiota and metabolite profiles used in **Paper I**, substantially improved the distinction of IBS patients from healthy subjects. Moreover, bowel habits were also reflected in this integrated intestinal microenvironment profile of IBS patients. Altogether, these results provide valuable insights into the pathophysiology of IBS and further confirm the complexity of the host–microbiome interactions in IBS. Further advanced knowledge of the intestinal microenvironment could enable the discovery of new biomarkers for diagnosis of disease, development of new therapeutic interventions, as well as predictive tools for therapy response. This however needs to be explored in more detail and remains a challenge for the future.

Considering the number of complex factors involved in IBS pathophysiology, it is perhaps not surprising that the currently available symptom-targeted treatment options have had somewhat limited success in improving the overall symptom burden in patients with IBS. The lack of success has led to the popularity of other treatment strategies, such as the use of compositionally complex Aloe gel. This thesis confirms that treatment with Aloe gel derived extract is safe and well tolerated by patients with IBS. Further, our results support Aloe gel extract as a treatment option, offering an effective improvement in the overall symptom severity in subsets of IBS patients (**Paper II** and **III**).

Disease heterogeneity is still a challenge in the management of IBS. Patients with different underlying pathophysiology and characteristic symptoms may benefit from different treatment strategies and hence the challenge is to provide the right treatment option to the right

patient. Aloe gel extract, attributed with a battery of therapeutic properties including potential prebiotic as well as immunosuppressive effects (**Paper II** and **IV**), may indeed be considered advantageous over pharmacological agents that have more precise modes of action and often accompanied by risk of adverse effects. It might also be worthwhile to identify subgroups of IBS patients, who are more likely to respond to specific therapeutic options. To second this, we have suggested a potential mechanistic relationship between treatment response to Aloe gel extract and the intestinal microenvironment. Moreover, we have demonstrated the predictive potential of gut microbiota and metabolite composition to identify IBS patient subsets who are likely to benefit from the Aloe extract treatment. These findings however warrant confirmation. Validation studies aiming at better stratification of IBS subsets based on pathophysiological traits, along with establishing biomarkers for predicting therapy response to treatment with Aloe gel extract, would enable us to improve personalised treatment of IBS patients.

Along with demonstrating the beneficial therapeutic effects of an Aloe gel derived extract in subsets of IBS patients, this thesis expands our knowledge of the complex and synergistic bioactive composition of Aloe gel. Despite similar commercial descriptions, Aloe gel extracts were found to differ not only in their standard phytochemical quality characteristics, but also differed notably in their metabolite composition, and in turn their potency of suppressive effect on immune cells (**Paper V**). Further, the quantitative and qualitative variation in bioactive composition of Aloe gel is known to be influenced by several factors including geographical location, seasonal changes and most importantly processing and extraction methods. Hence, we propose that it is not possible to assume that all Aloe gel extracts are similar, and efforts are needed to standardise commercial Aloe gel extracts. While each Aloe extract with its unique chemical composition may possess beneficial properties, consideration should be given to identify and link bioactive composition of Aloe extracts with the desired biological effects. Further research is however needed to understand better the bioactive composition of Aloe extracts. With future focus directed towards exploiting metabolomics to identify the “optimal” bioactive profile for Aloe gel, we hope to enable design and formulation of the next generation functional foods and therapeutic products with improved beneficial properties.

The focus of this thesis has been to evaluate an Aloe gel derived extract as a therapeutic option for IBS. Aloe gel, being attributed with several therapeutic properties, in particular its ability to reduce immune cell activity, may have immunosuppressive potential. Hence, Aloe gel extract may well be a prospective treatment option for various GI disorders and diseases, including inflammatory bowel disease, as well as inflammatory conditions in general. It would thus be worthwhile to validate and understand the potential immunosuppressive effects of Aloe gel extract, using multifaceted *in vitro* models such as co-cultures of epithelial cells and immune cells, human derived organoids models or gut-on-a-chip, as well as animal model studies, followed by controlled trials.

Altogether, further studies evaluating the pathophysiology and treatment options for IBS are warranted and will play an important role towards reducing the socioeconomic burden of this disorder. Therapeutic strategies like the use of Aloe gel derived extract, offering an effective improvement in the overall symptom severity in subsets of IBS patients without significant side effects, adds clinical relevance to this thesis. There is indeed a need to optimise the bioactive composition of Aloe gel extract associated with its therapeutic effects, to develop better defined products with beneficial properties. Nevertheless, the accumulated result from this thesis confirms an altered intestinal microenvironment in IBS patients and establishes the role of Aloe gel derived extract as a therapeutic option, which undeniably can play an important in role in disease management to improve patient outcome in IBS.

ACKNOWLEDGEMENTS

Finally, here we are! The most important section of this thesis where I attempt to express my sincere thanks to everyone who has contributed to this work and supported me, professionally and personally, through my doctoral studies. None of this would have been possible without the support of each one of you!

Thank you all ❤

My first thanks are for some very important supporters, whom I miss dearly! Gone but not forgotten!

Nanu, my dearest granddad, oh what I would do to present this thesis to you in person, to see the look of pride in your eyes and get your special hug in return. Thank you for always believing in me, for pushing me, supporting me and for the most important life lessons. I miss you so much and I wish you were here!

Stefan, you took me under your wings when I first started in the group. You were more than just a colleague, you were a mentor, a friend and father figure. I have learnt so much from you and I truly believe that your guidance and support helped me stay on and continue as a PhD student in the group. Thank you and rest in peace dear friend.

Dad, my father in-law, I am so honoured to have you as a role model. Your hard work, ideals, passion for your work and family are truly inspirational. A true gentleman, you are missed dearly. A special thanks to you for guiding me home.

As a PhD student the support of a good supervisor is essential for success, and I can without doubt say that I am fortunate to be blessed with the best group of Supervisors -

Lena Öhman, my main supervisor, you are an inspiration! I am grateful for your support and guidance through these last 7 years. Thank you for giving me this opportunity to be your PhD student. A lot has changed in these years, but what has remained constant is your exceptional leadership and strength. Thank you for being the most dedicated mentor and supervisor one could ask for. Thank

you for your patience, willingness to listen, for always believing in me and giving me the freedom to steer my research project. You are truly gifted with the ability to bring out the best in everyone around you!

Maria Magnusson, my co-supervisor, I have no idea what I would have done without your help and support for all things analytical and statistical! Your excitement for learning is contagious! I never thought I would be able to do half of the fancy multivariate analysis when I first started. Still learning but I have come a long way! Thank you truly, for all your time, effort, and guidance.

Fredrik Larson, my co-supervisor and colleague, my sincere thanks for all the discussions, constructive criticism and always helping me to widen my point of view. Thank you for pushing me to question more! You wear several hats, from researcher, our personal IT support, legislative support – an all-rounder help desk at the Calmino office! Thank you for everything I have learnt from you and thank you for helping me to make my own decisions.

Alastair Ross, my co-supervisor and metabolomics superhero, you have helped to improve and bring my research up to par with the future. Despite moving to another country and having new priorities, thank you for always finding time to guide me and support me with your expertise.

Magnus Simrén, my un-official supervisor, it has been an incredible honour to be able to collaborate and work with you. Your humble attitude, despite your immense knowledge and prestige in the field is inspirational. Still bewilders me on how you keep track of a million research projects and publications! Thank you for always listening and supporting!

Tobias Kisker, my boss and mentor, none of this would have been possible without you! You definitely set the bar up high as one of the most incredible bosses ever. Thank you for giving me this opportunity, for the never-ending support, encouragement and for letting me have the freedom and responsibility to drive this research project.

To my dream team of colleagues, group members- past and present, co-authors, collaborators, and everyone at the Department of Microbiology and Immunology! I have so much respect for you all and I am so thankful for everything I have learnt from each one of you.

Maria Sapnara, you have been a big sister and my constant supporter right from the start. You are a gem of a colleague and friend! Always helpful and super resourceful, with your crazy organizational skills, hidden math skills and the immense pride and care you put into everything you do. Thank you for the chats, lunches, and discussions! And thank you for your friendship and support over the years.

Cristina Iribarren and **Luiza Moraes-Holst,** you both are incredibly talented and inspiring young researchers. I am so glad I got this opportunity to share this great PhD adventure with you. It just wouldn't have been the same without you both! I'm glad I also got to collaborate with both of you and I know we will share many more amazing adventures together in the future!

Lujain Maasfeh and **Anna Forsberg,** even in the short time I have worked with you both, you have been so warm, supportive, and always willing to help. Thank you and good luck to you both for your research projects.

Former group members, **Sean Bennet** – I will always be grateful for the SIMCA crash course that you selflessly helped me with in your last working week. **Anna Strömbeck** and **Sofia Norlander**, thank you for your guidance and help during my first international conference and poster presentation. And for cheering me on during the last phase! **Johanna Sundin**, thank you for your help and collaboration! It has been a pleasure working with each one of you.

Otto Savolainen, thank you for a wonderful collaboration over the last several years and all your help with the metabolomic analysis and more. I look forward to more fun projects together in the future!

To all **my co-authors**, none of this would have been possible without you backing me all along the way! Thank you for your

contribution, valuable input, and proof reading each of the manuscripts. A special thanks to **Stine Störsrud** and **Lena Böhn**, who have been responsible for patient recruitment and without whom my research would not have been possible. Thank you for always being supportive and helpful. **Hans Törnblom,** thank you for being accommodating and willing to help me with my clinical questions.

To the amazing team at the **Mag-tarm lab**, thank you for all your hard work behind the scenes and for making the clinical studies possible. A special thanks to **Pernilla Jerlstad** and **Gunilla Näslin**, for being helpful with sample collection at the beginning of my research.

To my fellow PhD students and Post-docs at the Department of Microbiology and Immunology, thank you for your help and cooperation throughout these years. For creating such a friendly and learning environment. A special thanks to **Maria Öberg**, for being a great office mate, for help in the lab and for all the talks and laughs; **Andrew Broucher** and **Akshi Singla**, thanks for sharing your lab knowledge and always being welcoming to my million questions and doubts. Thanks also to research technicians **Karin Schön** and **Patrik Sundström,** for always being willing to help me despite your busy schedules.

To former Calmino colleagues, **Sara Alexandersson** and **Hozan Sadek**, thank you for all your administrative help with the studies and for your friendship.

To all the study participants, thank you, without you all there would be no research.

Finally, a thank you to my family and friends! I am truly blessed to have your unconditional support and love! Here is to you all!

Ma and **Pa**, my parents, there will never be enough words to describe how much gratitude and love I have for you both! Thank you for giving me this life and being my strength. For always supporting me with all my crazy plans, regardless of where in the world they took me. You both are the best! **Biks**, my little brother,

I love you and thank you for always being there for me and being my biggest supporter.

To my in-laws, **Mom**, **Dad**, and **Ricky**, thank you for opening your hearts to me when I first moved to Sweden and cheering me on through all my endeavours. A special thanks to mom and my sister-in-law **Soni**, for taking care of the kids and being my support during these very stressful last few months. I owe you one.

To my babies, **Akira** and **Aveer**, you both are my inner strength and the reason I will never give up! My biggest reason for happiness! My sunshine! You both make me so proud, and I love you both so much! And to my little papillon **Bella**, you have literally been my moral support, day after day and through the night, lying by my feet giving me company as I wrote my thesis.

Last but not the least, to my husband, my soul mate and my best friend, **Jimmy**. Thank you for being the wind beneath my wings, for always standing by me and for dreaming with me. For your patience and love. And of course, your mad graphic designing skills, the beautiful illustrations and the cover would not have been possible without your talented touch. I love you, thank you for being my person ❤

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All Figures and Box 1 have been created with Biorender and have the following agreement numbers:

Box 1: CT24H992ZS
Figure 1: HQ24KUG86K
Figure 2: HV24JONO8H
Figure 3: HH24GTRL9R
Figure 4: RB24JTN6D7
Figure 5: CO24K6HM48
Figure 6: AQ24KOZ6MU
Figure 7: ER24K6GYWP
Figure 8: EV24GURGP4
Figure 9: JZ24GUSDMC
Figure 10: HO24GUSWM5