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CHAPTER 9

Nutrition and Immunity

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9.1 The Immune System

As reviewing the essentials of the immune system would expand beyond the scope of this chapter, the reader shall be briefly reminded that the immune system is a complex ensemble of biological entities (cells, tissues, organs) and processes (inflammation, immune tolerance) that protect the integrity of the organism from external and internal threats.[1] External threats include micro-organisms (bacteria, viruses, parasites), their toxic products (exotoxins, endo-toxins, enzymes), and air- and food-borne allergens. The immune system also responds to severe trauma such as burns and physical injuries in a manner similar to the shock response that occurs with an overwhelming bacterial infection. Internal threats include:

- microorganisms that are otherwise normal commensals in the gut, respiratory and urogenital system and on the skin;
- abnormal cells (cancer); and
- the tendency of the immune system to attack itself (autoimmunity).

The two major functional components of the immune system are innate immunity and adaptive immunity. Innate immunity is by definition present prior to exposure to antigen and therefore can not be customized (adapted).

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It is phylogenetically conserved in all multicellular organisms.[2] The innate immune system is designed to recognize a few highly conserved motifs present in microorganisms. In contrast, the adaptive immune system is highly specific and increases both in specificity and magnitude with repeated exposure to antigen.[3] Unlike the innate immune system that recognizes pathogens through specific molecular markers, the cells of the adaptive immune system need to be educated to discriminate between "self" and "non-self".

9.2 Nutrition and Immunity

9.2.1 Macro- and Micronutrients

Nutritional status plays an important role in the functioning of the immune system.[4] Dietary proteins, carbohydrates and fats, as well as micronutrients (vitamins and minerals), interact with immune cells systemically in blood, regional lymph nodes and in the specialized gastrointestinal immune system.[5] The role of specific macro- and micronutrients in immune function has been extensively discussed in the literature in a dedicated issue of *Nutrition*. For example, Vanderhoof summarized the importance of carbohydrates, primarily seen as a source of energy, in immuno-nutrition.[6] Various amino acids such as glutamine,[7] arginine,[8] taurine[9] and sulfur-containing amino acids[10] have been reviewed in terms of their immunomodulatory properties. In addition, (poly)-unsaturated fatty acids have an impact on immune status and the role of ω-3 fatty acids has been specifically discussed by Alexander.[11]

Declines in both specific and non-specific immunity have been reported in association with under-nutrition and protein deficiency.[12] There is also considerable evidence that deficiencies of trace elements such as iron, zinc, selenium and copper, and vitamins A, B6, B12, folic acid, C, D and E are associated with impairments in immune function.[4,13–16] While natural food has the potential to supply most of the essential macro- and micronutrients, dietary supplements and/or enriched foods might be of great value in stress situations such as premature life, ageing or disease, or extreme conditions like exercise.

In terms of nutritional support and promotion of a healthy immunity, three levels of care are being pursued. The primary level consists of the provision of all key micro- and macronutrients to sustain immune cells and functions. The second level corresponds to the modulation of the immune system to appropriately respond to specific but broad areas of concern—an example would be proper management of inflammation. The tertiary level reflects nutritional interventions tailored to the individual immune disposition and situation and is hence part of preventive and personalized nutrition.[17]

9.2.2 Malnutrition, Under-nutrition and Immunity

The causal relationship between famine and pestilence has been known for millenna.[18] Malnutrition and infection are the two major obstacles for health,

development and survival worldwide, with malnutrition being the commonest cause of immunodeficiency worldwide.[14] While infection and malnutrition aggravate each other, nutrition does not impact all infections to the same extent.[19] Nutritional deficiency is commonly associated with impaired immune responses, especially cell-mediated immunity, cytokine production, secretory antibody response and affinity.[14] The proper consumption and absorption of micronutrients is essential for optimal immune responses (*e.g.* zinc, iron, selenium, vitamin A, pyridoxine, vitamin E).[20] But macronutrient balance also plays a role: animal proteins are generally superior to vegetable proteins in maintaining immunity. Moreover, there are subtle differences in immune responses of animals fed casein- and whey-based diets.[18] During periods of stress and illness, production of glutamine—the most abundant intracellular amino acid—is upregulated as branched chain amino acids are metabolized by skeletal muscle.[21] Glutamine is an important energy source for intestinal enterocytes and for rapidly proliferating cells such as immunocytes which react to challenges imposed by injury and illness.[22]

The immune system is undergoing permanent renewal and produces millions of immune cells daily. Immune cell renewal is elevated during infectious disease, and recovery depends on the rate of cell division between the invading microorganism and that of immune cells. The immune system uses both macro- and micronutrients involved in DNA, RNA and protein synthesis.[23] Thus, under-nutrition has a strong influence on the immune system at all ages but mainly in growing and aged humans, *i.e.* when the body's nutritional reserves are limited. At those life stages, under-nutrition is a major factor leading to immunodeficiency and thereby to higher infection rates.[23] This chapter therefore dedicates a special section to nutrition and immune function in newborns and infants as well as in elderly.

9.3. Mass Spectrometry in Immunology–Immunoproteomics

Immunoproteomics with a perspective from biomarker discovery to diagnostic applications was recently reviewed by Tjalsma *et al.*[24] The concept here is to refine, multiplex and accelerate mass spectrometry- and proteomics-based antibody analytics and diagnostics.

9.3.1 Mass Spectrometry in Immune-related Nutritional Intervention

In 2008 de Roos and McArdle presented their view on how to deploy proteomics as a platform for biomarker development in nutrition research.[25] This paper is probably the most comprehensive summary of (immune-related) nutritional studies as monitored by mass spectrometry and much of the work cited therein is either discussed directly below (with those being immune-relevant in a broader sense) or further on in this chapter. These studies are mostly

based on the classical proteomic approach, *i.e.* protein separation by two-dimensional (2D) gel electrophoresis followed by protein spot excision, in-gel protein digestion and mass spectrometric protein identification, the latter mainly deploying matrix-assisted laser desorption ionization (MALDI) mass fingerprinting but also liquid chromatography tandem mass spectrometry (LC-MS/MS).

For example, de Roos and McArdle demonstrated by MS-based proteomics that two structurally very similar dietary conjugated linoleic acid (CLA) isomers had divergent mechanistic effects on atherosclerosis development and insulin resistance in apoE 2/2 mice.[26] Equally relying on proteomics, their group could furthermore show that:[26,27]

- the consumption of dietary fish oil and *trans*10, *cis*12 CLA induced differential expression of long-chain acyl-CoA thioester hydrolase protein as an indicator of fatty acid β-oxidation in the liver; and
- the consumption of dietary fish oil, olive oil and *trans*10, *cis*12 CLA induced differential expression of adipophilin protein as an indicator of selective hepatic lipid accumulation and triglyceride secretion.

Arbones-Mainar and colleagues followed a proteomics-rooted approach to better understand the mechanisms by which olive oil fatty acids, or its minor antioxidant constituents, may affect hepatic metabolic pathways, oxidative stress and, eventually, atherogenesis.[28]

Mitchell *et al.* evaluated matrix-assisted laser desorption ionization time-of-flight (MALDI-ToF) mass spectrometry as a method for revealing protein biomarkers of an immune-modulating diet.[29] They identified α-2–HS glycoprotein B-chain as a biomarker of fruit and vegetable intake; during separate feeding periods, 38 participants ate a basal diet devoid of fruits and vegetables and a basal diet supplemented with cruciferous (broccoli) family vegetables. At the end of each seven-day feeding period, serum samples were obtained and abundant proteins were depleted. MALDI-ToF spectra were analyzed using peak picking algorithms and logistic regression models. Two significant mass peaks could classify participants based on diet (basal *vs.* cruciferous) with 76% accuracy. One peak was identified as the B-chain of α-2–HS glycoprotein, a serum protein previously found to vary with diet and be involved in immune function and insulin resistance.

A 2D gel- and MS-based proteome study published by the Daniel group also aimed to reveal protein biomarkers of dietary intake; they identified alterations in peripheral blood mononuclear cell (PBMC) proteins of healthy males ingesting flaxseed for a week. PBMCs from the same study subjects were also exposed *ex vivo* to physiological concentrations of enterolactone (a metabolite produced from dietary lignans by colonic microflora) to assess whether similar effects on the proteome could be observed as those caused by dietary flaxseed. A fairly robust change in 16 PBMC proteins was observed upon flaxseed consumption. Four out of these 16 protein changes were similar to those found in blood mononuclear cells exposed *ex vivo* to enterolactone:[30]

- enhanced levels of peroxiredoxin;
- decreased levels of long-chain fatty acid β-oxidation multi-enzyme complex proteins; and
- levels of glycoprotein IIIa/II.

However, most of the more traditional nutritional interventions reported to date that aim to improve immune condition have not (yet) deployed mass spectrometry to assess status, bioavailability and metabolism of the nutrient(s) or dietary antigens of interest and their effects at molecular level.[31,32] Rather, few- or single-point read-outs were performed with classical assays based for example on high performance liquid chromatography (HPLC) and internal standards.[33] Immune studies are often based on mononuclear cells cultured in standardized systems applying a chosen stimulus and a single endpoint. The analytical methods to assess immune response to nutritional or other stimuli have been reviewed[34] and encompass mainly immune cell-based assays (*in vitro* models or *ex vivo* samples), cytokine measurements, flow cytometry and delayed-type skin hypersensitivity testing.

While it is reasonable to link nutrient intake with immunological outcomes, it would be desirable to make measurements in between the very beginning and the very end of an intervention study, namely the known quantity of the nutrient as orally taken in and physiological endpoints. One of the rare examples of such an investigation is the study by Woelkart *et al*. who looked at bioavailability and pharmacokinetics of *Echinacea purpurea* and their interaction with the immune system.[35] *Echinacea* is a widely used herbal remedy for the prevention and treatment of the common cold. In order to compare the bioavailability of alkamides (the main lipophilic *Echinacea* constituents) from liquid and tablet preparations of *E. purpurea* in humans and to study the effects on *ex vivo* stimulated blood cells, a randomized, single dose, crossover study was performed. Liquid chromatography coupled to electrospray ionization ion-trap mass spectrometry (ESI-IT-MS) was used to determine the content of alkamides in serum. Both *E. purpurea* preparations led to the same effects on the immune system according to the concentration of pro-inflammatory cytokines.

Modern nutritional intervention studies should try to follow the fate of the nutrient at molecular level, and then add bioavailability and bioefficacy data to intake information in order to establish a better causality between the nutrient and its effect.[36] Mass spectrometry is ideally suited for nutrient bioavailability, bioefficacy and metabolism studies thanks to the intrinsic sensitivity and information-rich spectra it can deliver for almost all organic compounds.[36] Apart from these assets for targeted nutrient and metabolite analysis—especially in the highly sensitive and selective single reaction monitoring (SRM) and multiple reaction monitoring (MRM) mode as performed on triple quadrupole (QqQ) machines for proteomic purposes[37,38]—MS can empower nutritionists "to be prepared for the unexpected": it can elucidate nutrient metabolism in a holistic way and enables metabolite discovery.[39] The latter aspect is of particular importance to molecular nutritional research because the desired health

effects of nutrients as enriched or "remixed" in functional food must not be compromised by less desirable side effects. In other words, health promotion through adapted nutrition "must get everything right".

9.3.2 Mass Spectrometry in Discovery of Immune Markers and Targets

Markers to measure immunomodulation in human nutrition intervention studies have been reviewed by Albers *et al.*[40] These markers do not descend from "omic" approaches but rather reflect targeted measurements of biomolecules or read-outs from cellular assays, typically performed in (pre-) clinical settings. The role of proteomics deployed for the discovery of biomarkers in gastrointestinal diseases has been outlined by Song and Hanash,[41] who describe protein microarrays, mass spectrometry-based proteomic tools and guidelines for biomarker development. The authors state that inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) represent diseases for which biomarkers are still pending and that proteomics may help in identifying them. Within IBD, better markers are needed to distinguish between Crohn's disease and ulcerative colitis, and to improve diagnosis and prediction of therapy.

A review by Purcell and Gorman[42] on mass spectrometry-based studies of immune responses discusses the role of proteomics in:

- elucidation of the cytotoxic T lymphocytes;
- T cell—B cell co-operation and antibody secretion;
- defining targets of T cell immunity;
- discovery of T cell epitopes;
- analysis of antigen presenting cell (APC) surface proteins; and
- sequencing of major histocompatibility complex (MHC)-bound peptides.

Addressing a more specific immune context, Weingarten *et al*. discussed the application of mass spectrometric protein analysis to biomarker and target finding for immunotherapy.[43] Their article focuses on regulatory T cells that play a central role in maintaining the immunological balance and inhibiting T cell activation both *in vivo* and *in vitro*. The enhancement of suppressor cell function is suggested as a target for immunotherapeutic treatment of immune-mediated disorders such as multiple sclerosis and Crohn's disease. The proposed method of choice to elucidate the still unclear effector functions of regulatory T cells is differential proteomics of human and murine T cell populations. Applying such an approach, the same group at Protagen AG plus other colleagues have assessed the human CD4+ CD25+ regulatory T cell proteome and identified galectin-10 as a novel marker essential for their anergy and suppressive function.[44]

Cereals are the most important nutritional component in the human diet. Food-induced allergic reactions to these substances therefore have serious implications and exhaustive diagnosis is required. Such diagnosis is still difficult

because of the incomplete knowledge about major cereal allergens. In particular, few food-induced allergic reactions to maize have been reported and no information on the allergenic proteins is available. Having observed several anaphylactic reactions to maize, Pastorello *et al.*[45] aimed to identify major maize allergens and their cross-reactivity with other cereals, as well as to peach, because the majority of patients also reacted to *Prunoideae* fruits. Twenty-two patients that showed systemic symptoms, positive skin prick tests and serum-specific immunoglobulin E (IgE) antibodies after maize ingestion were selected. The IgE reactivity pattern was identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. The major allergen identified was then purified by HPLC and characterized by mass spectrometry.

Proteomics in humans with auto-immune diseases has been reviewed by Chan and Utz[46] with a discussion of associated effects in inflammation. The diagnostic and therapeutic potential of glycans in inflammation has been assessed by Dube *et al.*,[47] with particular emphasis on glycosylation changes resulting from chronic inflammation. The review emphasizes the challenge of glycomics and glycoproteomics (*i.e.* the analogue of proteomics at glycan and glycoprotein level); glycan biosynthesis is not template- but enzyme-dependent (glycosyltransferases and glycosidases form glycans on lipid and protein scaffolds), and therefore renders the global, quantitative analysis of glycan expression a daunting task. However, the functions of glycans found at sites of chronic inflammation are relatively well-defined compared to, for example, cancer-associated glycans and their changes.

Aguiar *et al.* presented a mass-spectrometry based, clinically relevant assay for the quantification of C-reactive protein (CRP), a well-established and clinically relevant marker of inflammation.[48] Exact quantities of two synthetic ^{13}C-labeled CRP tryptic peptides were added as internal standards to the sample prior to chemical treatment, tryptic digestion and LC-MS quantification *ex vivo*. The method was applied to the quantification of urinary CRP from a study of drug-induced nephrotoxicity.

9.4 Proteomics of Intestinal Epithelial Cells

A series of recent publications focused on intestinal epithelial cells (IECs) harvested *ex vivo* or cultivated *in vitro* for functional studies of inflammation-related gut disorders. An *in vitro* proteome analysis of intestinal epithelial cells has demonstrated the cytokine-induced synthesis of proteins involved in the amplification of the inflammatory response such as heterogeneous nuclear ribonucleoprotein JKTB, interferon-induced 35-kDa protein proteasome subunit LMP2 or arginine metabolism-related enzymes (tryptophanyl- tRNA synthase, indoleamine-2,3-dioxygenase and arginosuccinate synthetase).[49]

Shkoda *et al.* presented protein expression profiles in the intestinal epithelium from patients with inflammatory bowel disease.[50] The scientific rationale behind this work was IEC function alteration shown to be critical in initiation

and progression of chronic intestinal inflammation in the genetically susceptible host. The 2E MALDI-MS proteomic study compared ileal and colonic primary IECs from patients with Crohn's disease, ulcerative colitis to those from non-inflamed controls. Among the 21 proteins found regulated relative to the normal IECs, nine reached statistical significance and the most pronounced changes were detected for programmed cell death protein 8 and annexin 2A. Moreover, changes in expression of proteins implicated in signal transduction, stress response and energy metabolisms were found in IBD patients. A further interesting observation was the differential expression of the signal transduction regulator Rho GDIα, an inhibitor of cell cycle progression and mediator for pro-apoptotic mechanisms. The induction of Rho GDIα has been associated with the destruction of epithelial cell integrity and increase in intestinal permeability.

The same group deployed a proteomics approach to investigate the role of interleukin-10 (IL-10) to block endoplasmic reticulum stress in IECs.[51] Primary IECs from IL-10 $-/-$ mice and IBD patients revealed increased expression levels of the glucose-regulated endoplasmic reticulum stress protein (grp)-78 under conditions of chronic inflammation. Primary IECs from both inflamed IL-10 $-/-$ mice and IBD patients demonstrated activated endoplasmic reticulum stress responses in the intestinal epithelium. One anti-inflammatory mechanism of IL-10 seems to root in the inhibition of inflammation-induced ER stress response by modulating ATF-6 nuclear recruitment to the grp-78 gene promoter. The authors concluded that loss of regulation with respect to endoplasmic reticulum responses in the epithelium may contribute to the pathogenesis of chronic intestinal inflammation.

The Déchelotte group compared the proteomes of human intestinal epithelial HCT-8 cells *in vitro* after glutamine supplementation under non-stimulated and inflammatory[52] and apoptotic conditions.[53] Glutamine (Gln) is an important amino acid for the enterocytes. It promotes intestinal growth and maintains gut structure and function, especially during inflammation, where the endogenous Gln stores are rapidly depleted. Increased gut proteolysis, in addition to a reduction of mucosal protein synthesis, may lead to mucosal atrophy in the absence of adequate nutritional supply. Two-dimensional gel and MS-based proteomics were utilized to characterize glutamine effects on the human intestinal epithelial HCT-8 cell line under non-treated and pro-inflammatory conditions.[52] Under non-stimulated conditions, 24 proteins were differentially expressed in response to Gln. Half of these proteins are implicated in protein biosynthesis or proteolysis and 20% in membrane trafficking. Under pro-inflammatory conditions, 27 proteins were up- or downregulated by Gln. Among these, 40% are involved in protein biosynthesis or proteolysis, 16% in membrane trafficking, 8% in cell cycle and apoptosis mechanisms, and 8% in nucleic acid metabolism.

The influence of glutamine on intestinal proteome expression in apoptotic conditions was also studied in HCT-8 cells.[53] The pharmaconutritional effects of glutamine were determined under 2 mM (physiological concentration) and 10 mM (pharmaconutritional concentration) conditions. Among 1800 protein

spots revealed in both conditions, 28 proteins were differentially expressed in response to an increased glutamine concentration in the culture medium, with 24 identified by mass spectrometry. Of these, 34% are involved in cell cycle and apoptosis, 17% in signal transduction, and 13% in cytoskeleton organization. The proteome-based findings are relevant to establish the effects of glutamine on intestinal barrier function and inflammatory responses, and to open new mechanistic approaches to optimize nutritional support under specific conditions.

Intestinal epithelial cell protrusions referred to as microvilli or brush border membranes (BBMs) are specialized in digestion, uptake and transport of nutrients from intestinal lumen into the circulation. Native protein complexes in murine intestinal BBMs have been recently described.[54] The blue native PAGE (BN-PAGE) technique combined with LC-MS/MS was recruited to separate and identify native digestive protein complexes in BBMs in order to better understand the physiology and pathology of digestion and absorption. Twenty-three distinct protein complexes were found and their protein composition was determined. Overall, 55 individual proteins were identified including peptidases, enzymes of carbohydrate metabolism, membrane transporters, cytoskeletal proteins, chaperones and regulatory enzymes.

9.5 Inflammation and Nutrition

9.5.1 Definition of Inflammation

Inflammation is a basic process whereby tissues of the body respond to injury. Inflammation has been described as purposeful, timely, powerful and, as a consequence, also as dangerous, if resolution is not initiated.[55] The normal outcome of the acute inflammatory programme is successful resolution and repair of tissue damage, rather than persistence of the inflammatory response.[56] Emerging evidence suggests that a co-ordinated programme of resolution initiates during the first few hours after an inflammatory response begins. Natural resolution of inflammation is a highly complex, multifactorial and tightly controlled process driven by removal of the initial stimulus, decrease in pro-inflammatory mediators (mainly cytokines, chemokines), elimination of damaged and inflammatory cells, and promotion of repair.[57,58] Although inflammation is essential for tissue homeostasis, prolonged inflammation is a hallmark of many chronic diseases such as inflammatory bowel disease and auto-immunity. Moreover, chronic inflammation has been shown to be implicated in critical conditions such as atherosclerosis, arthritis, cancer and asthma—all leading to tissue destruction, fibrosis and impairment or loss of organ function.

9.5.2 Inflammation and Nutrition

Pro-inflammatory cytokines and oxidant molecules produced during the inflammatory response following infection and injury may be beneficial or

detrimental to the patient, depending on the amounts and contexts in which they are produced. Aberrant or excessive production is implicated in inflammatory disease. Systems exist for the control of cytokine production and oxidant actions. The former include the hormones of the hypothalamo–pituitary–adrenal axis, acute phase proteins and endogenous inhibitors of interleukin (IL)-1 and tumour necrosis factor (TNF). The latter encompass endogenously synthesized antioxidants (*e.g.* glutathione) and dietary antioxidants (*e.g.* tocopherols, ascorbates and catechins). Nutrients change cytokine production and potency by influencing tissue concentrations of molecules implicated in cytokine biology (for a review see ref. 59). Monounsaturated fatty acids and ω-3 polyunsaturated fatty acids (PUFAs) suppress TNF and IL-1 production and actions, while n-6 PUFAs exert the opposite effect. Low antioxidant intake results in enhanced cytokine production and effects. The anorexia that follows infection and injury may be purposeful to permit substrate release from endogenous sources to support and control the inflammatory process. Therefore, prior as well as concurrent nutrient intake co-determines the outcome of the inflammatory response.[59]

Figure 9.1 displays schematically how inflammation can be managed by nutritional means. Probiotics, TGF-β caseinate, antioxidants and lipids influence intestinal inflammation, gut integrity, tissue repair and oxidative stress. Antioxidants and lipids also modulate acute phase proteins and the glutathione (GSH)-based redox system in the liver. Moreover, free amino acids feed into muscle catabolism and can influence the oxidative stress in the muscle.

The Daniel group applied a 2D gel- and MALDI mass spectrometry-based strategy to reveal proteomic biomarkers of dietary response in human PBMCs;

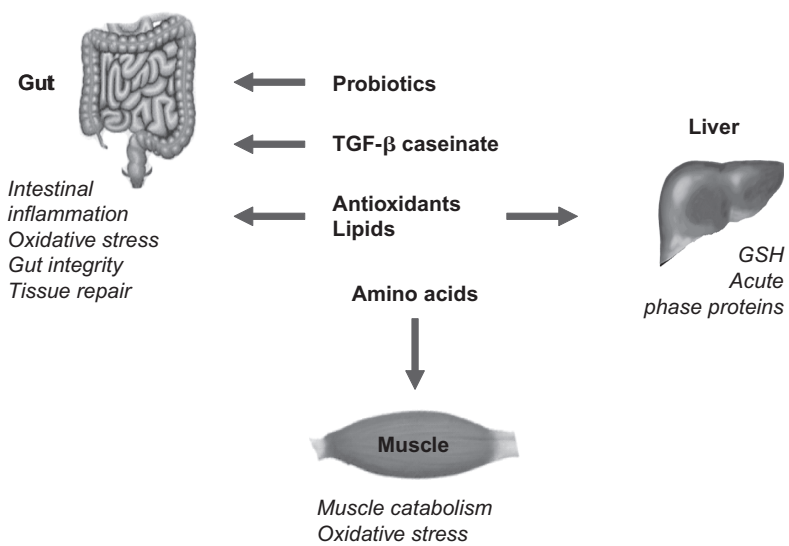

Figure 9.1 Managing inflammation by nutritional means.

postmenopausal women received a supplementation with an isoflavone extract for eight weeks.[60] Twenty-nine proteins—including several involved in the anti-inflammatory response—showed altered expression in the mononuclear blood cells following the soy-isoflavone intervention. As no overall anti-inflammatory activity of the soy intervention was observed at the level of clinically relevant inflammation markers in plasma, the PBMC proteome was suggested to be a more sensitive target to detect inhibition of inflammatory processes and to possibly respond earlier than those plasma markers classically assessed.

9.5.3 Intestinal Inflammation

Celiac disease and Crohn's disease are prototypic disorders of gastrointestinal mucosal immune function.[61] Crohn's disease is characterized by chronic inflammation of the gastrointestinal tract and associated with multiple genetic mutations, at least one of which has been clearly implicated in innate immunity. Moreover, the disease appears to involve abnormal immune responses to gut microbiota.[61] Celiac disease is a disorder of the small intestine characterized by chronic inflammation of the mucosa caused by loss of tolerance to dietary antigens. Among the associated cofactors identified are antigenic peptides in wheat, rye and barley diets. Most patients have complete remission after elimination of these cereals. The immune system cannot afford to err on the side of caution because failure to mount effective and vigorous immune responses will be exploited by pathogens. This can be exemplified by celiac disease, in which the high prevalence of HLA-DQ2 in the general population suggests an evolutionary advantage of this allele against infection, even when facing the negative effects of the coincidental affinity of gluten peptides for HLA-DQ2 to cause celiac disease. Because of these conflicting interests of the immune system, it may be unrealistic to prevent chronic inflammatory (gut) diseases and hence new treatments should be based on a molecular understanding of the disease.[62]

Inflammatory bowel disease arises in part from a genetic predisposition through the inheritance of contributory genetic polymorphisms. These gene variants may be associated with an abnormal response to normal luminal bacteria. In view of these findings, Ferguson *et al.* presented a nutrigenetic review on IBD and dietary exposure/intervention.[63] *In vivo* models of inflammatory bowel disease elucidate important mechanisms of chronic inflammation. Roy *et al.* applied nutrigenomics to an animal model of inflammatory bowel disease.[64] However, their investigation of the effects of diets enriched with eicosapentaenoic acid (EPA) and arachidonic acid (ARA) remained at transcriptomic level and did not deploy mass spectrometry.

The Bendixen group has presented one of the few mass spectrometry-based *in vivo* proteomic studies in the context of intestinal inflammation.[65] Acquisition of passive immunity by endocytosis of intact immunoglobulins (Ig) from colostrum is critical for preventing intestinal and systemic diseases in neonatal mammals. Therefore the group compared proteome patterns of healthy and

inflamed gut tissues harvested from pre-term piglets to investigate the effect of inflammation on acquisition of passive immunity. A clear difference in the 2D gel electrophoretic protein patterns between healthy and inflamed intestinal tissues was revealed, suggesting that inflamed tissues failed to absorb and transfer Ig from colostrum to epithelial cells. Mass spectrometry identified isoforms of the IgA and IgG heavy chain and Ig κ and λ light chains as being absorbed by healthy intestinal tissues and indicated that colostrum protein uptake in the porcine gut is a selective process deranged in inflamed pre-term intestine.

Widening the context of intestinal inflammation, the mechanisms of salicylic acid modulating potentially pro-cancerous activity in the colon were investigated in a rat model of oxidative stress using MS-based proteomics.[66] Supplementation of salicylic acid resulted in expression changes of 55 cytosolic proteins extracted from the distal colon. The functions of these proteins related to redox balance, protein folding, protein transport, energy metabolism and cytoskeletal regulation.

9.5.4 Holistic Views of Inflammation

Innate immunity is the main mechanism for immediate responses to infection and cellular injury. Elements of innate inflammation are conserved in all multicellular organisms and predate the evolution of the adaptive immune system.[55] The recognition of "non-self" in combination with so-called "danger signals" (derived from bacteria or damaged cells) and the subsequent inflammatory response to this recognition comprises effector mechanisms of both innate and adaptive immunity.[67–69] In westernized countries, most infectious diseases of the gut are largely condemned, while gastrointestinal food allergies and idiopathic inflammatory conditions have dramatically increased: we seem to now have inflammation without infection. The absence of gut infection may have disturbed the balance between the normal bacteria that colonize the healthy gut and the mucosal immune system.[62]

Activation of the adaptive immune system is essential to mount (antigen)-specific, mainly Th1-driven, responses and to generate regulatory T cells. The latter are key players in the control of inflammation either by direct cell contact and/or secretion of immuno-regulatory cytokines such as IL-10 and/or TGF-β.[70,71]

In a network-based analysis of systemic inflammation in humans, Calvano *et al.* showed the genome-wide transcriptional response to systemic administration of bacterial lipopolysaccharides (LPS).[72] Transcriptomic analysis of PBMCs demonstrated the temporal activation of gene clusters implicated in innate immune responses, but also interconnected genes involved in cell cycle control, apoptosis, cytoskeleton protein synthesis and mitochondrial energy production. This example highlights the self-limiting character of the innate immune response in healthy conditions. By contrast, chronic inflammation manifests itself by a deregulation of functional modules interrelated in

physiological conditions. The erosion of such functional networks documents the high degree of plasticity of immune cells to rapidly adapt to changing conditions such as injury, inflammatory insults or infections with the overall goal of re-establishing homeostasis. The complexity and flexibility of immuno-regulatory networks highlights the need for their holistic analysis, to which the omics sciences with chip-based transcriptomics and mass spectrometry-rooted proteomics are now beginning to contribute.

9.5.5 Mass Spectrometry in Inflammation

Despite the fragmentary understanding of inflammation networks and only emerging contributions from mass spectrometry-driven, holistic proteomic studies, inflammation is already a major target for dietary intervention with bioactive food ingredients. Several nutritional strategies, including n-3 PUFA, antioxidants vitamins, plant flavonoids, prebiotics and probiotics are being explored with the aim of dampening chronic inflammatory processes. However, nutritional studies still largely deploy cell cultures and animal models, and the potential of extrapolating to human nutrition remains limited. Therefore, more studies in human subjects and holistic, non- or minimally invasive readouts are urgently required. Mass spectrometry clearly has to expand its role here as the tool of choice to comprehensively interrogate easily accessible body fluids such as blood,[73,74] urine,[75] saliva,[76] tears[77] and nasal fluid.[78]

Although nutritional studies have focused on therapy of inflammatory conditions and appropriate nutrition may lower the risk of such conditions, strong molecular evidence of this effect is currently lacking.[79] This said, naturally occurring ''nutraceuticals'', especially antioxidant bioactives such as plant phenols, vitamins, carotenoids and terpenoids, have revealed benefits by tempering sustained inflammation accompanying chronic disease.[80] Targeted genes directly involved in inflammation encompass cyclo-oxygenase-2 (COX-2), TNF-α, IL-1, phospholipase A2, 5–lipoxigenase (LOX) and inducible nitric oxide synthase (iNOS). Almost a thousand plant extracts were screened for potential modulators of COX-2 expression.

9.6 Allergy and Nutrition

9.6.1 Definition of Allergy

The term allergy is understood as the overshooting, IgE-mediated response of an organism towards an allergen.[81] Atopy means, in more general terms, the disposition for the development of allergic symptoms.[82] Allergy can manifest in various forms such as neurodermitis (affecting the skin) and asthma (affecting the respiratory tract).[81] Accordingly, allergens can be classified according to their channels of interaction with the host: airborne allergens invade the respiratory system, food allergens are taken up by the gastrointestinal tract and contact allergens act through the skin. The channel of invasion does not

necessarily correspond to the locus of allergy manifestation: some food allergens can, for example, provoke allergic reactions in the respiratory tract.[81]

Allergy is mainly governed by Th2 cells, which express the interleukins (ILs) 4, 5, 6, and 13. Autoimmunity is controlled by Th1 cells expressing IL-2, IL-12, IL-18, interferon (IFN) α and γ, as well as TNF-α and TNF-β. T-regulatory cells (Tregs), secreting IL-10 and TGF-β, control the balance between Th1 and Th2 cells, and regulate in this way the specific allergen response and maintain normal immunity.[83]

9.6.2 Allergy is a Public Health Issue

Over the last 25 years, the occurrence of allergy has dramatically increased and the World Health Organization (WHO) has declared it as one of the epidemics of the 21st century. The so-termed "hygiene hypothesis" proposes a paucity of microbial exposure during childhood as one of the causes of the allergy epidemic in Western countries.[84] Western infants show a delayed acquisition of several gut microbes and a reduced turnover of strains in the microbiota, indicating exposure to a low variety of environmental bacteria.[85]

9.6.3 Allergy Markers

Most of the to-date identified 20 or more allergy-associated genetic markers are rather indicators of inflammation than of allergy. Kornman *et al.* described a nutrigenomics strategy to better understand the associations between genetic variations, the susceptibility to inflammation and the nutritional intervention potential.[86] Important allergy markers accepted to date are IL-10,[87] TGF-β, TLRs,[88] PD-1 and CTLA-4.[89] Yet, specific IgE levels are successfully used as indicators for an allergic condition. Roughly 150 genes are suspected to be linked with the multiple phenomena of the three allergic diseases—atopic dermatitis, hay fever and asthma.[90] Today, only few gene trait links for allergy susceptibility and pre-disposition are established, one of which is the identification of a susceptibility locus for asthma-related traits on chromosome seven revealed by a genome-wide scan in a Finnish founder population.[91] Figure 9.2 shows a large-format 2D gel proteome display of PBMCs.

9.6.4 Food Allergy

Food allergy is an adverse reaction to food or food additives with an underlying immunological mechanism. Its incidence in young children and among adults is approximately 1.3% and 0.3%, respectively. Parental history of atopy is a significant causal factor and exposure to common allergenic foods in infancy increases risk. For these reasons, exclusive breastfeeding and maternal avoidance of peanut, egg, fish and dairy products during lactation have been recommended and shown to reduce the occurrence of food allergy.[93] The

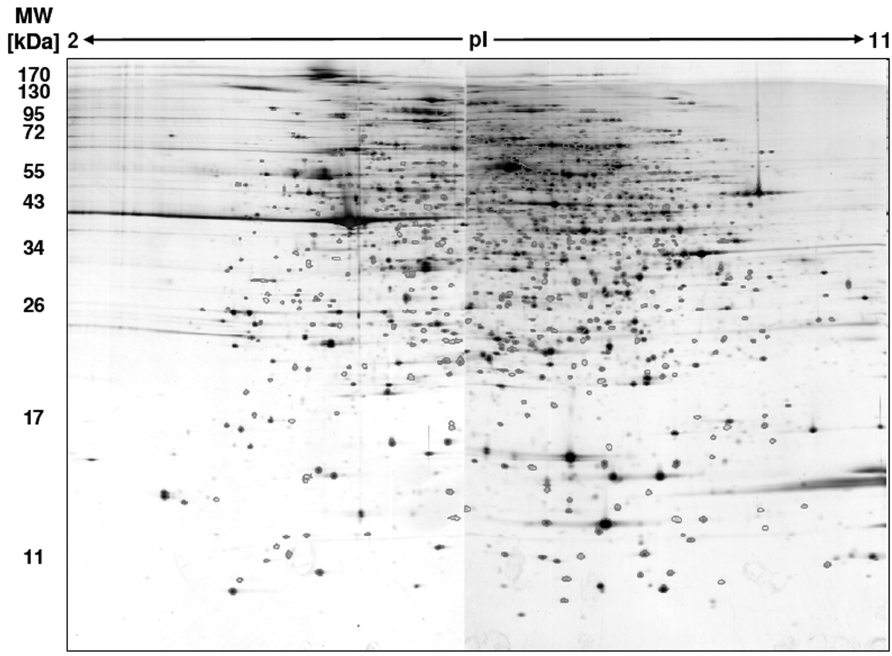

Figure 9.2 Large-format 2D gel proteome display of PBMCs. PBMCs represent an intensely assessed immune cell population because they are available in large numbers and by minimally invasive means, *i.e.* blood sampling. The gel spans a pI range from 2 to 11 and a Mr range from >10 to *ca.* 200 kDa. In this particular study on allergy biomarkers,[92] typically 2000 protein spots were detected per gel, ~1200 spots were matched, and ~700 spots were matched and quantified across all technical and biological replicates.

consequences of breastfeeding and early nutrition are discussed in more detail in a subsequent, dedicated section.

Many food allergens have been identified and these stimuli are often structurally well characterised, typically by mass spectrometry of the implied proteins and peptides. This source of risk necessitates detecting and monitoring (potential) allergens before, during and after food processing.[94] A list of the ten most sensitising proteins has been proposed. Although this may vary from country to country, these proteins basically derive from egg, fish, shellfish, milk, soy, wheat, peanuts, tree nuts, citrus fruits and sesame seeds. Most of these food allergens are glycoproteins and most in the range 14–40 kDa.[93] These physicochemical characteristics render them ideal analytes for mass spectrometry and proteomics, with their power to identify, sequence and quantify proteins and post-translational modifications such as glycosylation, and to differentiate between protein isoforms.[95,96] Cow milk protein allergy is still an increasing problem for infants. MALDI-ToF-MS is well suited to address this

concern, as it has been extensively used to characterize allergens in cow milk.[97] Further examples for mass spectrometric efforts in protein allergen characterisation are:

- identification of the hazelnut 11–S allergen;[98]
- discovery of sesame seed allergens;[99] and
- immunological analysis of shrimp allergens.[100]

In contrast to the advanced level of understanding about allergen structures, the molecular mechanisms deciding on a normal or an allergic reaction (*i.e.* the consequences of allergen exposure for the host) are incompletely understood. Prediction of allergy risk and onset is mainly based on family history data. In terms of individual disposition, genetics and environmental influence are difficult to dissect. The environmental imprinting as a counter-player of the genetic determination is most important during pregnancy, the weaning period and in early childhood (see also Section 9.8 on "Early Nutrition").

Circulating leukocytes (or PBMCs) are good objects for proteomic studies of an individual's immune status.[101] They are available in large amounts from healthy and diseased subjects, can be harvested by minimally invasive means and cultured under near-physiological conditions. Moreover and importantly, PBMCs have a normal active metabolism.[102] Differential proteomics of PBMCs require a sufficient number of biological and technical replicates in order to understand the pronounced and meaningful inter-donor variability in protein profiles and to discern it from the undesired experimental variations.[103]

9.7 Gut Mucosal Immunity, Intestinal Microbiota and Probiotics

9.7.1 Gut Mucosal Immunity

Second to the respiratory tract, the gastrointestinal tract is the body's largest tissue boundary with a surface area of *ca.* 300 m^2.[21] It interacts with nutrients, exogenous compounds and gut microbiota, and its condition is influenced by these environmental factors and host genetics. Intestinal functions such as digestion, nutrient absorption, barrier integrity, motility and mucosal immunity are all under complex regulatory control.[104] Moreover, the intestine is the primary immune organ of the body represented by the gut-associated lymphoid tissue (GALT) exerting innate and acquired immunity. Three constituents are in permanent contact and dialog with each other—the microbiota (see Section 9.7.2), the mucosal barrier and the local immune system.[105] Figure 9.3 shows how mass spectrometry and omics tools can come into play to investigate the intestinal immune system; at the gut barrier, enteric bacteria enter into crosstalk with the mucosal immune system.[55]

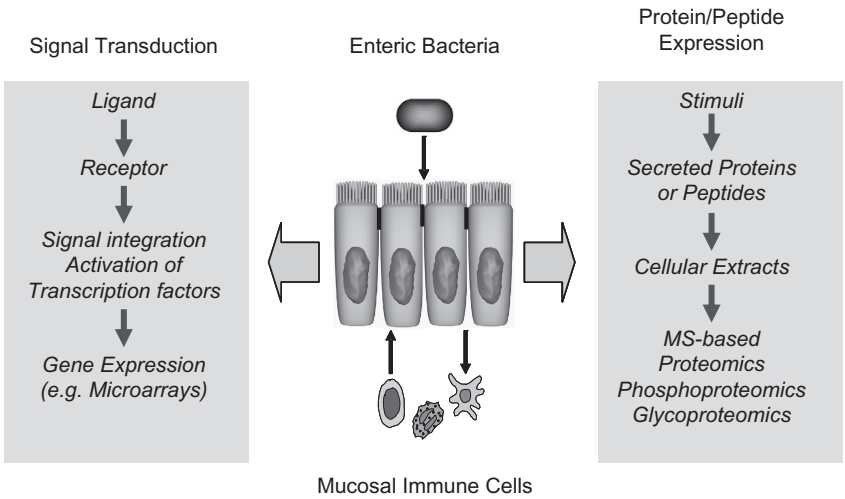

Figure 9.3 At the gut barrier, enteric bacteria enter into cross-talk with the mucosal immune system.[58] This interface can be sampled by Omics techniques to elucidate signal transduction and protein/peptide secretion. Signal transduction can be globally assessed by gene microarrays whereas MS-based proteome analysis can reveal immune biomarkers.

9.7.2 Gut Microbiota

Humans and other mammals are colonized by a vast, complex and dynamic consortium of microorganisms. In fact, adult humans are numerically more prokaryotic than eukaryotic: estimates are that 90% of our cells are microbial, whereas only 10% are human.[106] The impact of these indigenous microbial communities on our physiology is likely to be most pronounced in the intestine because this organ harbours the vast majority of our bacteria. Microbial densities in the proximal and middle small intestine are relatively low but increase dramatically in the distal small intestine ($\sim 10^8$ bacteria per g of luminal contents) and colon (10^{11}–$10^{12}\,g^{-1}$).[106]

Gut microbes conduct a multitude of biochemical reactions and can be collectively thought of as a metabolically active ''organ.'' This metabolic entity plays a critical role in nutrition, degrading a number of dietary substances that are otherwise non-digestible.[107] One ''raison d'être'' for this metabolically active microbial society is to harvest energy from nutrients, especially carbohydrates.[108]

The microbiota in the adult human body consists of an enormous biomass of $>100\,000$ billion bacteria spread over >400 different species which generate intense metabolic activity, mainly in the colon, and play an important physiological role in the host.[105] The microbiota has a major impact on gastrointestinal and mucosal immune functions. Colonization of the gut by commensal bacteria has been shown to alter intestinal physiology of the host by modulation of genes implicated in nutrient absorption, mucosal defences and

xenobiotic metabolism.[109,110] It is now established that one of the essential functions of the colonic microbiota is its ability to resist colonization by any new strain of bacteria from the exterior.[105]

9.7.3 Probiotics

Probiotics are live microbial food and feed supplements, which beneficially affect the host by improving its intestinal microbial balance.[111] Recent evidence indicates that probiotics (*e.g. Streptococcus thermophilus* and *Lactobacillus bulgaricus*) may influence both systemic and gut-associated immune responses.[112] Some probiotics enhance while others suppress immune responses.[113] Probiotics seem to act through stimulating regulatory T cells, which can activate both these responses.[114] Most of the immunobiological effects of probiotics are likely to take place in gut-associated lymphoid tissue, including Peyer's patches, in the small intestine. Due to comparable numbers of probiotic and resident bacteria at that location, probiotics may compete with luminal microbiota more successfully than in the colon, which is already heavily populated with indigenous bacteria. Furthermore, the cross-talk between probiotics and the small intestine may be different from that in the colon and it may be age-dependent.[114]

Certain probiotic strains are reported to control inflammation,[115] reduce the risk of allergy[116] and restore gut comfort in chronic painful conditions.[117] Probiotics have furthermore been reported to promote tolerance,[118] maintain intestinal immune homeostasis[119] and prevent atopy.[120] Lactobacilli are one of the most frequently used strains of probiotic bacteria in the management of gastroenteritis, inflammatory bowel diseases[121] and atopic diseases.[122] These probiotic bacteria are also suggested to regulate immunity and promote mucosal tolerance, which is in part mediated by Treg cells.

Clinical applications of probiotics for adults encompass inflammatory bowel disease, irritable bowel syndrome, *Helicobacter pylori* gastritis and improvement of intestinal transit. For infants and children, probiotics have been administered to fight acute diarrhoea, acute childhood constipation, *Helicobacter pylori* gastritis and intestinal bacterial overgrowth. For a review of these studies see Walker *et al.*[114]

Bifidobacteria, a class of probiotics, are important components of the human intestinal microbiota, in which they occur at concentrations of 10^9–10^{10} cells per gram of feces,[123] and of fermented milk products, to which they are added mainly because of their health promoting activities.[124] The entire genome of *Bifidobacterium longum* has been sequenced.[125] Champomier-Verges *et al.*[126] reviewed mass spectrometry-based proteomic studies dealing with lactic acid bacteria. Two research interests were pursued. The first aimed to establish a systematic protein map for taxonomy and function assignment of proteins. The second axis focused on proteins, the synthesis of which is induced by various environmental factors. Such studies may give new insights for the usefulness of bacteria in human health and in the struggle against bacterial pathogens.

Tolerance to digestive stresses is one of the main factors limiting the use of microorganisms as live probiotic agents. These effects as well as technological stresses (heat, pressure, shear) are major factors affecting viability and thus the efficiency of probiotic microorganisms in food products. Proteomic analyses have shown that pre-treatment of the probiotic strain *Propionibacterium freudenreichii* with a moderate concentration of bile salts greatly increased its survival rate in subsequent challenges.[127,128] Marvin-Guy *et al*. have published a rapid identification of stress-related fingerprints from whole cells of *Bifidobacterium lactis* using MALDI-MS.[129] Guillaume *et al*. have found markers of heat shock resistance by comparing the proteomes of two *Bifidobacterium longum* strains.[130] The proteomic data were compared to and corroborated by a related gene expression study.[131]

9.7.4 Prebiotics and Synbiotics

Prebiotics have been defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of probiotic bacteria in the colon and thus improve host health.[132] As a logical extension of the probiotic concept backed up by prebiotic ingredients, combined symbiotic approaches are being pursued[133] that aim to define the right combination of beneficial gut bacteria and food ingredients which foster the growth and activity of the latter.[134] For example, one study evaluated the effects of six weeks' consumption of a symbiotic product containing *Lactobacilli* and fructo-oligosaccharides (FOS) on intestinal microbiota, self-reported intestinal function and the immune function of generally healthy adults.[135] Although no differences in self-reported improvement were found with treatment of mild gastrointestinal symptoms present at baseline, there was a significant improvement overall in symptoms and in motility in the symbiotic group compared with the placebo group. Intestinal microbiota did not change as a result of symbiotic consumption.

Liquid chromatographic separation coupled to mass spectrometric detection is the platform of choice to structurally characterize prebiotics and study their metabolic fate. This has been pursued, for example, in the context of (milk) oligosaccharides by Lebrilla *et al*.[136,137] and is further discussed in the milk-related section of this chapter. LoCascio *et al*. utilized a HPLC-chip ToF mass spectrometric approach to glycoprofiling of bifidobacterial consumption of human milk oligosaccharides (HMOs).[137] HMOs were separated from pooled human breast milk samples and several bifidobacterial strains grown on them. The oligosaccharides were isolated and purified from the supernatant and analyzed on a high-resolution ESI Q hybrid Fourier transform ion cyclotron resonance (FTICR) mass spectrometer and on a HPLC-chip-ToF-MS system. LoCascio *et al*. demonstrated strain-specific, preferential consumption of small-chain glycans secreted in early human lactation.

Apart from this latter application, mass spectrometry remains under-deployed for such purposes. However, as a key analytical element of functional

genomics, mass spectrometry is expected to serve in unravelling interdependencies between prebiotics and probiotics, commensal host–bacterial relationships in the gut[109] as well as naturally occurring and designed symbiotic relationships.[138–140]

9.7.5 Gut Ecology

NMR- and MS-based metabolomics is uniquely suited and increasingly deployed to capture the metabolic interplays between the host metabolism, and its symbiotic and parasitic microorganisms in the colonic flora.[141,142] The characterization and mathematical modelling[143] of this metabolic cross-talk between microbiota and host should result in a better understanding of the long-term health consequences associated with an optimal or impaired microbiotic activity. Metabolomics bears also great potential for investigating the effects of dietary ingredients that target the colonic flora such as probiotics (mainly lactobacteria) or prebiotics (mainly soluble fibres).

It is extremely difficult to simulate the complex bacterial–mucosal immune interaction using *in vitro* models. Nicholls *et al.* deciphered metabolic events associated with acclimatization of germ-free rats to standard laboratory conditions.[144] Martin and colleagues modelled transgenomic metabolic effects consecutive to the inoculation of non-adapted human faecal flora in a mouse model.[141] In order to elucidate gut microbial effects under relevant conditions, animals with *a priori* sterile gastrointestinal tract and monocolonised with probiotics are now used as a suitable model, especially the gnotobiotic mouse,[109,110,145] but also germ-free piglets.[146] The latter model was deployed to investigate the effects of bacterial colonization on the porcine intestinal proteome by mass spectrometry.[146] Small intestinal protein expression patterns in gnotobiotic pigs maintained germ-free or mono-associated with either *Lactobacillus fermentum* or non-pathogenic *Escherichia coli* were studied. A common reference combined with stable isobaric tags (iTRAQ) for relative protein quantification revealed that bacterial colonization differentially affected proteolysis, epithelial proliferation and lipid metabolism, which corroborated studies of other germ-free animal models.

Our molecular understanding of how members of the intestinal microbiota degrade complex polysaccharides derives from studies of *Bacteroides thetaiotaomicron*, a prominent and genetically changeable component of the normal human and mouse gut. Colonization of germ-free mice with *B. thetaiotaomicron* (Btheta) has shown how this anaerobe modifies many aspects of intestinal cellular differentiation/gene expression to benefit both host and microbe.[108,145,147] The Btheta proteome encompasses specific functions for polysaccharide acquisition and hydrolysis, and an environment sensing system.[148] The same group undertook a combined gene expression and GC-MS-based metabolomics study; GC-MS was performed on the standard mouse chow diet and on the total caecal contents recovered from sterile and

Btheta-colonized animals. Sonnenburg *et al*. found that the predominant *in vivo* responses to Btheta-association reflected glycobiome activation:[149]

- Btheta glycosyl hydrolases correspond to the most prominent sugars in the environment;
- Btheta prefers the monosaccharides that can be metabolized most efficiently; and
- Btheta is able to degrade both plant- and host-derived polysaccharides.

Compared to the gut commensal and probiotic Btheta, *Bifidobacterium longum,* a minor member but a commonly used probiotic, has a more restricted glycan-degradation machinery but a larger repertoire of transporters[108] suggesting that *B. longum* may directly benefit from Btheta's "upstream" polysaccharide degradation.[114] To address this latter hypothesis, the Gordon group colonized germ-free mice with *B. thetaiotaomicron* and *B. longum*. Simultaneous whole genome transcriptional profiling of both bacterial species in their gut habitat and of the intestinal epithelium, combined with mass spectrometric analysis of habitat-associated carbohydrates, revealed that *B. longum* expanded the diversity of polysaccharides targeted for degradation by *B. thetaiotaomicron* (*e.g*. mannose- and xylose-containing glycans) and induces host gene expression involved in innate immunity. Although the overall transcriptome expressed by *B. thetaiotaomicron* when it encounters *B. longum* in the caecum depends upon the genetic background of the mouse, Btheta's expanded capacity to utilize polysaccharides occurs independently of host genotype and is also observed with a fermented dairy product-associated strain, *Lactobacillus casei*. Hence, this gnotobiotic mouse model provides a controlled case study of how a resident symbiont and a probiotic species mutually adapt their substrate utilization, and illustrates both the generality and specificity of the relationship between a host, a component of its microbiota and intentionally consumed microbes.[149]

The pioneering work by Gordon *et al*. documents two things:

- gut ecology is extremely complex and it takes an ecosystem approach to understand the health impact of the intestinal microbiota including probiotics;[145] and
- genomics as well as mass-spectrometry-rooted proteomics and metabolomics are the tools of choice to provide holistic mechanistic insights into this host–microbe cross-talk.[138]

9.8. Early Nutrition and Immunity

9.8.1 Immune Development around Birth

Profound immunological changes occur during pregnancy, involving a polarization of T helper (Th) cells towards a dominance of Th2 and regulatory T

cell effector responses in both mother and foetus. This situation is important to maintain pregnancy through avoidance of the rejection of the immunologically incompatible foetus. During the third trimester of human pregnancy, foetal T cells are able to mount antigen-specific responses to environmental and food-derived antigens and antigen-specific T cells are detectable in cord blood in virtually all newborns indicating *in utero* sensitization. If the neonatal immune system is not able to downregulate the pre-existing Th2 dominance effectively, an allergic phenotype may develop.

Important changes occur also around birth so that the neonate's immune system becomes competent and functional, and the gut is colonized with bacteria. Mucosal immune response is primed at birth and responses generated at this time support specific immunity in later life.[150] Infants are born with a practically sterile gut, which is rapidly colonized. The predominant source for initial colonization is the maternal flora, followed by the environmental flora.[105] Exposure to bacteria during birth and from the mother's skin, and the provision of immunological factors in breast milk are among the key events that promote maturation of the infant's gut and the gut-associated as well as systemic immune systems. The maturing small intestine of the newborn is initially exposed to a large number of colonizing bacteria acquired while passing through the birth canal. In the absence of mature intestinal function (mucus production, peristalsis, *etc.*), large numbers of bacteria colonize the small intestine. This contrasts with the mature intestine, in which large numbers of colonizing bacteria are only present in the distal ileum, caecum and colon. The early exposure of the small intestine to colonizing flora is an important step in the appropriate maturation of mucosal immune system.[114] A compositional comparison of the intestinal microflora between healthy and allergic infants, for example, showed that the latter had fewer Lactobacilli and Bifidobacteria, but more Clostridia and coliform bacteria.[151] Probiotics can downregulate, namely minimize, an IgE-mediated allergic response[152] and are involved in re-establishing oral tolerance to food allergens even after sensitisation.[153]

The introduction of infant formula and solid foods exposes the baby to novel food antigens and affects the gut flora. Nutrition is the source of antigens to which the immune system must become tolerant. Nutrition provides factors, including nutrients, that themselves might modulate immune maturation and delivers compounds that influence the intestinal microbiota, which in turn affects antigen exposure, immune maturation and responses. Through these mechanisms, nutrition early in life influences and even "programmes" later immune competence, *i.e.* the ability to both mount an appropriate immune response upon infection, and develop a tolerogenic response to "self" and to benign environmental antigens.[154]

9.8.2 Milk as the Ideal Early (Immuno-) Nutrition

Figure 9.4 shows the major human milk proteins. Human milk mainly consists of caseins, α-lactalbumin, lactoferrin, albumin and various immunoglobulins.

These predominant proteins account for >99% of the milk protein mass. However, the remaining <1% encompass a complex blend of bioactive proteins and peptides, which is still far from being fully exploited at both analytical and functional level.

The mammary gland has a large metabolic potential including the large-scale synthesis of milk proteins, carbohydrate and lipids. Peng and colleagues carried out a proteomic analysis of mammary tissue to discover proteins affecting lipid metabolism.[155] Unfractionated microsomes from lactating bovine mammary tissue were separated with 1D-PAGE and identified by LC-ESI-MS/MS, yielding 703 proteins including 160 predicted transmembrane proteins. More than 50 proteins were associated with cellular uptake, metabolism and secretion of lipids. This database provides a proteomic view of the metabolic potential of the mammary gland. In a related study, the Smith group characterized the human mammary epithelial cell (HMEC) proteome;[156] they reported on a cysteinyl peptide enrichment (CPE) approach, which improved both protein sequence and overall proteome coverage. The combined analyses of HMEC tryptic digests with and without CPE resulted in ~4300 different proteins with an estimated 10% gene coverage of the human genome. CPE contributed roughly an additional 1000 relatively low abundant proteins, resulting in a further increase in proteome coverage. Almost 1400 proteins were observed with increased sequence coverage. Comparative protein distribution analyses revealed that the CPE method is not biased with regard to protein molecular weight (Mr), isoelectric point (pI), cellular location or biological function.

Secretory immunoglobulins, lysozyme, interferon and growth factors are known to confer immunological advantages to breast milk. Inhibition of bacterial pathogens and permissive growth of a protective colonic microbiota are partly promoted by breast milk.[157] Besides providing nutrition to the newborn, milk also protects the neonate and the mammary gland against infection. Breast-fed newborns have been shown to experience a lower incidence of gastrointestinal infections and inflammatory, respiratory and allergic diseases. This finding has been attributed to a diversity of protective factors in breast milk. One specific biological activity in mother's milk was reported to be the one of soluble CD14 (sCD14).[158,159] The study indicated a central role for sCD14 during bacterial colonization of the gut and suggested sCD14 to be involved in modulating local innate and adaptive immune responses, thus controlling homeostasis in the neonatal intestine. Another related study revealed an interaction between soluble Toll-like receptor 2 (sTLR2) and sCD14 in plasma and milk, proposing the existence of a novel innate immune mechanism regulating microbially induced TLR triggering.[160] A particular fraction of human milk, generated by a special chromatography based on restricted access material (RAM), was characterized by 2D LC-MS/MS in order to elucidate the protein composition and to discover novel molecules that potentially interact with sCD14.[161]

Differences were observed in the composition of intestinal bifidobacterial species depending on the type of milk fed. While *B. breve* is one of the

	[mg / 100 mL]
caseins	**250**
α-lactalbumin	**250**
lactoferrin	**175**
albumin	**50**
IgA	**125**
IgG	**4**
IgM	**3**

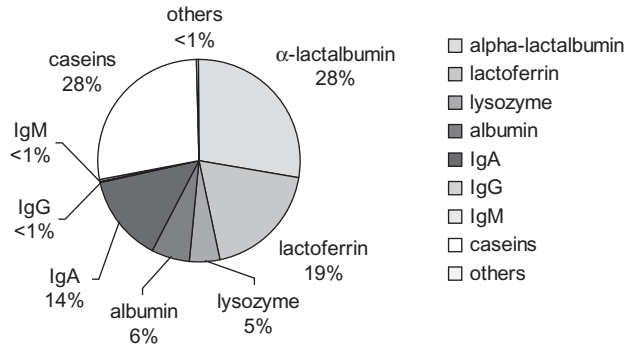

Figure 9.4 Major human milk proteins.

predominant species of the gut microbiota of breast-fed babies, *B. catenulatum* and *B. adolescentis* are characteristic of that of formula-fed infants.[162] Puertollano *et al.* assessed the differential effect of the bifidobacterial species identified in the intestinal microbiota of breast-fed and formula-fed infants on cytokine production by PBMCs.[163] The effects of different bifidobacterial species were analyzed individually and in combinations representing their proportions in infants under both feeding types. The effects of breast-fed and formula-fed bifidobacterial species combinations on cytokine production were not significantly different. These results suggest that the presence or absence of particular bifidobacterial species and the overall composition of the bifidobacterial population in the infant gut could be key factors defining the immunomodulatory effect of the gut microbiota in early life.

9.8.3 Mal-/Under-nutrition and Immune Imprinting

Moore summarized the context of nutrition, immunity, and the fetal and infant origins of disease in developing countries.[164] For instance, events in early life strongly influence the adult survival prospects of rural Africans:[165] nutritional status depends highly on season in rural settings as can be found in Gambia. There, individuals born during periods of seasonal nutritional deprivation are more susceptible to mortality from infectious diseases in adult life. A permanent negative imprinting of the immune system during fetal growth caused by malnutrition appears to be a likely explanation.[165] A related study on long-term effects of perinatal nutrition was published by Ghattas *et al.*, who investigated T lymphocyte kinetics of young Gambian men depending on the nutritional status of their mothers at the time of their birth.[166] This study rooted analytically in stable-isotope labelling of T cell subsets combined with GC-MS and revealed the astonishing finding that, in healthy young Gambian men, T lymphocyte homeostasis is extremely robust regardless of perinatal nutritional compromise.

9.9. Mass Spectrometry in the Analysis of Milk

9.9.1 Milk and Health

Milk is a rich source of bioactives beneficial for human health. It is the only nutrition that has co-evolved with mankind and is therefore particularly relevant and suited to support healthy growth and development of the neonate and infant, including the maturation and maintenance of a balanced immune system.[167] Milk bioactives derive from the protein and peptide,[168] the lipid[169] and the oligosaccharide complement[136] of milk from diverse mammalian species.

The protein complement of human milk roughly splits into caseins and whey with a 50 : 50 weight/weight ratio. Bovine milk consists of 80% caseins and 20% whey proteins;[168] a review of bioactive peptides and proteins present in milk and dairy products has been published by Severin and Xia.[168] For example, caseins serve as ion carriers and precursors of bioactive peptides, whereas whey proteins have major functions in immune modulation and defence.[170]

Specific milk fractions have been shown to alleviate immune deregulations like inflammation and osteoarthritis but without addressing molecular mechanisms or directly identifying ingredients responsible for the observed effects. More recently, milk peptides and hydrolysates moved into the focus of studies of bioactive compounds. Peptides with opioid, antihypertensive, antithrombotic, immunomodulating and metal-binding activities have been described in the review by Severin and Xia.[168]

9.9.2 Milk Analytics

Due to its analytical versatility and power for structure elucidation and quantification of larger biomolecules, mass spectrometry has developed into the major contributor to comprehensive biomolecule characterisation in milk, nowadays known under the more recently coined terms milk proteomics/peptidomics, lipidomics and glycomics. Casado *et al.* recently released a comprehensive review of the protein/peptide, lipid and carbohydrate complement of human and animal milk as assessed by various mass spectrometric approaches.[171] Fong *et al.* presented an update on bovine whey protein fractionation and characterisation by proteomic techniques including chromatography, gels and mass spectrometry.[172]

Traditionally, milk and its fractions have been assessed in terms of composition, physicochemical properties and biological functions, and mass spectrometry is largely contributing to various areas of milk and dairy research (reviewed in ref. 173 and ref. 174). These studies include identification of milk protein variants and glycoforms, falsification of milk with non-dairy ingredients and identification of peptides in dairy products. Moreover, mass spectrometry has become an indispensable technique for the quality assessment of milk- and dairy based products (reviewed in ref. 175).

Most relevant to the scope of this chapter, Smolenski *et al.* have characterised host defence proteins in milk deploying a dual proteomics

approach;[176] they applied both classical 2D gel electrophoresis and MALDI-ToF-MS, and a shotgun LC-MS/MS technique to bovine skim milk, whey and milk fat globule membrane (MFGM) fractions. Milk from peak lactation as well as during colostrum formation and mastitis was analyzed. In total, 2903 peptides were detected by LC-MS and 2770 protein spots by 2D gel electrophoresis. From these, 95 distinct gene products were identified, comprising 53 identified through direct LC-MS/MS and 57 through 2D gel electrophoresis and mass spectrometry. The latter stemmed from a total of 363 spots analyzed, with 181 being identified. At least 15 identified proteins are involved in host defence.

9.9.3 Breast Milk and Substitutes

Human breast milk is still considered the gold standard for neonate and infant nutrition. D'Auria *et al.* have undertaken a proteomic evaluation of different mammalian species for the potential to optimize infant formulas classically based on bovine milk and to recruit possible alternative sources for human breast milk substitutes.[177] Goat, horse, donkey and water buffalo milk were compared to human and bovine samples by 2D gel electrophoresis and mass spectrometry. In a milk protein hydrolysis study, the release of β-casomorphin-5 (BCM5) and β-casomorphin-7 (BCM7) was investigated during simulated gastrointestinal digestion (SGID) with pepsin of bovine β-casein variants, commercial milk-based infant formulas and experimental infant formulas.[178] β-Casein variants were extracted from raw milks derived from Holstein–Friesian and Jersey cow breeds. Identification and quantification of BCMs involved HPLC coupled to tandem MS. In view of the ongoing debate on β-casein health benefits, these data from SGID of infant formulas provide information for the evaluation of the potential bioactivity of bovine milk protein used in the manufacturing of infant formulas.[178] In another infant milk product-related study, lactosylated proteins of infant formula powders were investigated and this resulted in the identification of α-lactalbumin with five lactosylated peptides.[179] These may serve as protein markers to detect chemical modification induced by milk processing and/or storage.

Purification and characterization of novel peptide antibiotics from human milk has been described by Liepke and co-workers.[180] Digestion of human milk by infants was simulated by using pepsin under acidic conditions to generate peptides with antimicrobial activity. LC fractionation followed by MALDI-MS analysis allowed the identification of novel casein- and lactoferrin-derived fragments, which inhibited the growth of bacteria and yeasts.

9.9.4 Colostrum

Human colostrum (*i.e.* early breast milk) is an important source of protective, nutritional and developmental factors for the newborn. Colostrum (and other fractions) from different species were investigated mass spectrometrically by several groups mainly resulting in protein catalogues of these samples[177,181]

including low-abundance proteins.[182] A recent profiling of human colostrum revealed, after immunodepletion of high abundant milk proteins, a list of 151 low abundant proteins, 83 of which have not been previously reported in human colostrum or milk.[183]

9.9.5 Milk Fat Globule Membrane

The milk fat globule membrane (MFGM) is derived from the apical region of the mammary gland epithelial cells and budded off around the milk lipids, the latter being secreted by the mammary gland cells. MFGM is considered to be similar to any other eukaryotic cell membrane and accounts for 2–4% of the total human milk protein content.[184] MFGM may therefore contain—in addition to molecules previously described to be associated with this membrane (mucins, lactadherin, adipophilin, CD 36 and butyrophilin)—other factors to date thought to be exclusively found in cellular membranes. Figure 5 shows the physical organisation and major membrane-anchored proteins of the milk fat globule membrane.[185]

Nutritional and technological aspects of MFGM material have been recently reviewed.[185] Argov *et al*. recently investigated the particle size-dependent lipid content of human milk fat globules by Raman spectroscopy and reviewed milk fat globule composition, size and distribution.[186] Despite the large body of knowledge about its unusual biochemical structure, little is known about the physiological function of MFGM for the nursing infant. As such, it bears great potential for the identification of new proteins in milk and the exploitation of these proteins for dairy product development.[187] While MFGM proteins have a low nutritional value in classical terms, they play important roles in cellular processes and defence mechanisms for the newborn. MFGM is a particularly rich source of bioactive peptides and proteins.[188] Smolenski *et al*. compared the host defence proteome in MFGM, whey and skimmed milk by direct LC-MS/MS and 2D gels plus MALDI-ToF-MS.[176] Milk samples from peak lactation, during colostrum formation and during mastitis were analyzed resulting in a total of $\sim$2900 peptides detected by LC-MS and $\sim$2800 protein spots resolved by 2D gel electrophoresis. Of these, 95 distinct gene products were identified, comprising 53 unravelled by the shotgun and 57 through the gel approach. At least 15 proteins were found to be involved in host protection against infection.

Several Italian groups teamed up to chart the human colostral MFGM proteome and established a 2D gel electrophoresis MFGM protein database.[184] Reinhardt and co-workers analyzed the composition of bovine MFGM by 1D-PAGE and nano LC-MS/MS proteomics and identified 120 proteins, 71% of which were membrane associated.[189] Pursuing an iTRAQ-based shotgun proteomic approach, these authors also investigated developmental changes in the bovine MFGM proteome during the transition from colostrum to milk.[190] They identified 138 proteins, with 26 being upregulated and 19 downregulated in day 7 MFGM compared with colostral MFGM. Mucin-1 and mucin-15 were upregulated in MFGM from day 7 milk. Adipophilin, butyrophilin and

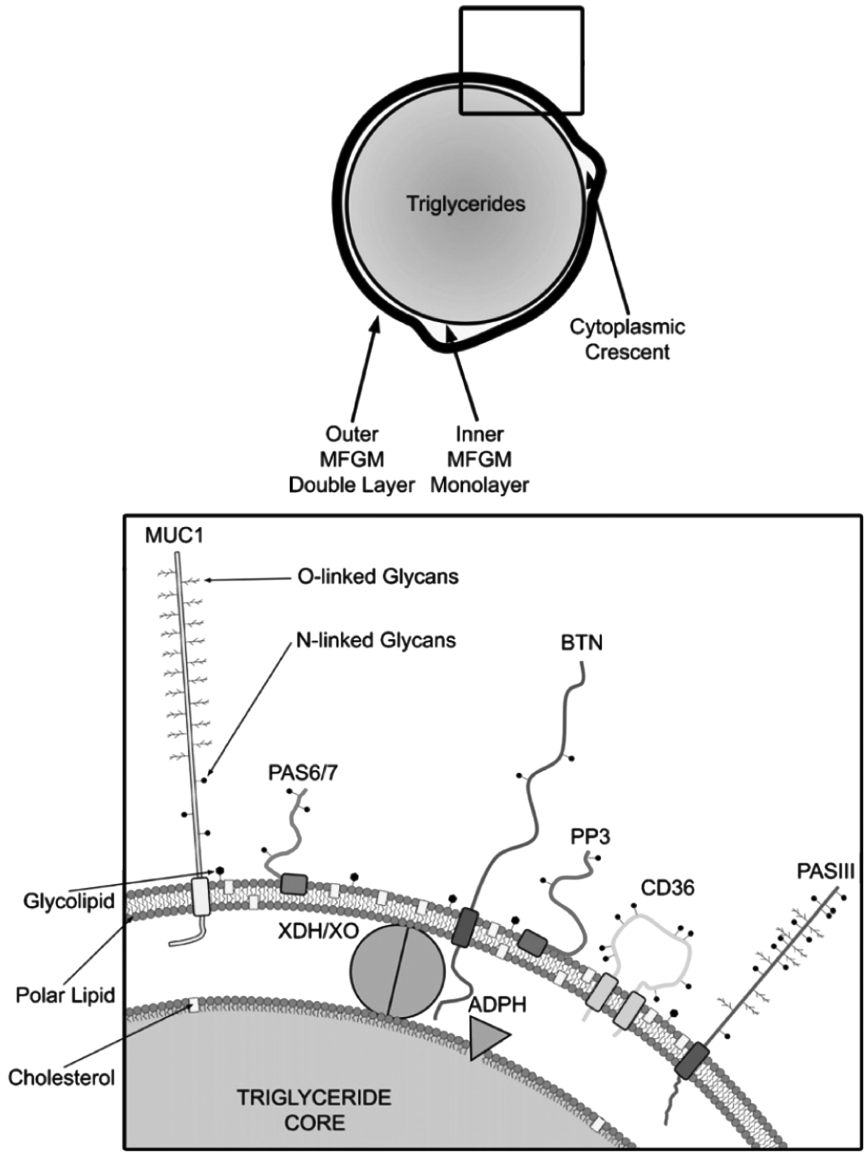

Figure 9.5 Physical organisation (top) and zoom (below) major membrane-anchored proteins of the milk fat globule membrane (MFGM): lactadherin (PAS6/7) protects from viral gut infection; mucin-1 (MUC1) and mucin-15 (PASIII) protect against rotavirus infection; adipophilin (ADPH) is involved in fatty acid/triacylglyceride (TAG) uptake and transport; fatty acid binding protein (FABP); xanthine dehydrogenase (XDH/XO) is bactericidal and anti-inflammatory; butyrophilin (BTN) suppresses multiple sclerosis; and platelet glycoprotein 4 (CD36). Modified from ref. 188.

xanthine dehydrogenase were individually upregulated in day 7 MFGM compared to the colostral fraction. Proteins associated with lipid transport, synthesis and secretion were also upregulated in day 7 MFGM. In contrast, apolipoproteins A1, C-III, E and A-IV were downregulated in day 7 MFGM relative to colostral MFGM.

Affolter *et al*. took a more targeted proteomic approach to compare MFGM-enriched milk fractions from different sources.[191] Applying a strategy based on multiple reaction monitoring (MRM) and labelled, proteotypic peptides as internal standards, they quantified seven bioactive MFGM proteins in absolute terms, namely lactoferrin, α-lactoglobulin, mucin, fatty acid binding protein (FABP), lactadherin, xanthine dehydrogenase/oxidase, adipophilin and butyrophilin.

Wilson *et al*. elucidated differences in sugar epitopes on human and bovine MFGM.[192] Their data indicate that human milk may provide different innate immune protection against pathogens compared to bovine milk as evidenced by the presence of Lewis b epitope (a target for *Helicobacter pylori*) on human but not bovine MFGM mucins.

9.9.6 Milk Protein Modifications

A considerable effort of MS-based milk protein research has focused on the elucidation of post-translational modifications such as glycosylation and phosphorylation.[193,194] For instance, Kjeldsen *et al*. aimed to completely characterize post-translational modification (PTM) sites in the bovine milk protein PP3 by tandem mass spectrometry with electron capture dissociation (ECD) as the last stage.[195] In their approach, termed ''reconstructed molecular mass analysis'' (REMMA), the molecular mass distribution of the intact protein is measured first, revealing the extent and heterogeneity of modifications. The protein is then digested, peptides are separated by reversed phase (RP) HPLC and analyzed by Fourier transform mass spectrometry (FTMS). Vibrational excitation (collisional or infrared) or electron capture dissociation of peptide ions provides protein identification. When a measured peptide molecular mass suggests the presence of a post-translational modification, vibrational excitation determines the type and structure of the modification, while ECD determines the PTM site. Chromatographic peak analysis continues until full sequence coverage is reached, after which the molecular mass is reconstructed and compared with the measured value. Agreement indicates that the PTM characterization is complete. This procedure has been applied to the bovine milk PP3 protein containing 25% modifications by weight and yielded all known modifications (five phosphorylations, two *O*- and one *N*-glycosylation) as well as a previously unreported *O*-linked NeuNAc-Hex-[NeuNAc]HexNAc group at Ser^{60}. FTMS-based REMMA can serve as the basis for high-throughput, high-sensitivity PTM characterization.

Protein alterations[196,197] and covalent complexes between milk proteins (*e.g*. caseins and β-lactoglobulin)[198] have also been investigated. Casein micelles, for

example, formed by interaction of milk caseins and calcium phosphate, maintain a supersaturated calcium phosphate concentration in milk, providing the newborn with sufficient calcium phosphate for the mineralization of the rapidly growing calcified tissues. The phosphorylation state of caseins plays an important role in the interaction with calcium phosphate and thereby in the organization of the micelles.[199] Other trace elements associated with milk proteins, such as iron in lactoferrin,[200] are important constituents to provide the newborn with essential nutrients. Inductively coupled plasma (ICP) MS has the analytical potential for ''element-tagged'' proteomics of milk, resulting in quantitative information on multi-element distribution patterns in different milk sources.[201]

9.9.7 Cryptomes

Bioactivities of peptides encrypted in major milk proteins are latent until released and activated, *e.g.* during gastrointestinal digestion or food processing. Bioactive peptides can be produced *in vivo* following intake of milk proteins. Moreover, the proteolytic system of bacterial species used in fermentation (*e.g.* yogurt, cheese) can contribute to the liberation of bioactive peptides or precursors thereof.[202] A wide range of proteins contain concealed functional units that can be liberated to generate novel bioactivities. Autelitano *et al.* term this ''hidden'' subset of peptides, residing within the proteome, the ''cryptome'',[203] and it is suggested to represent a vast array of cryptic peptides or ''crypteins'', with manifold bioactivities that can be liberated from the parent protein *via* proteolytic cleavage. Mass spectrometry is the obvious and powerful tool to study those ''cryptomes''.

9.9.8 Milk Allergens

Despite all these health beneficial effects of milk proteins, milk is also a source of protein allergens. Natale and co-workers characterized milk allergens by 2D gel electrophoresis immunoblotting and mass spectrometry.[204] The serum from 20 milk-allergic subjects was searched for major cow's milk allergens followed by MALDI-ToF-MS identification of the proteins. Zeece *et al.* investigated the effect of high-pressure treatment on *in vitro* digestibility of β-lactoglobulin (β-LG) under simulated gastric conditions using pepsin.[205] The proteomic study, based on one-dimensional (1D) gels and MALDI-ToF-MS, concluded that high-pressure treatment increased the digestibility of β-LG and represents a promising processing technology for reducing the allergenicity of known allergens in a wide variety of food materials.

9.9.9 Human Milk Oligosaccharides

Human milk is often the sole dietary source for the first few months in life. It contains all the nutrients necessary for the infant to thrive, but also ingredients

that may provide health benefits beyond those of traditional nutrients. Human milk oligosaccharides (HMOs) represent an abundant and diverse component of human milk, even though they have no direct nutritive value to the infant.[136] One litre of mature human milk contains approximately 5–10 g unbound oligosaccharides, and >130 different HMOs have been identified. Both their high amount and structural diversity are unique to human milk. Only trace amounts of these oligosaccharides are present in mature bovine milk and, as a consequence, in bovine milk-based infant formula. The potential health benefits of HMOs uncovered over the years may affect breast-fed infants both locally and systemically.[206] A recent hypothesis proposes that they could be substrates for the development of intestinal microbiota and the mucosal immune system.[137,207] Kunz and Rudloff recently reviewed the health-promoting aspects of milk oligosaccharides with reference to:[207]

- (prebiotic) oligosaccharides as growth factors for Bifidobacteria;
- anti-adhesion effects of milk oligosaccharides;
- systemic effects;
- leukocyte-endothelial interactions;
- plant-derived prebiotic oligosaccharides (PBOs) *vs.* HMOs;
- linkage specificity between monosaccharides in HMOs and PBOs; and
- benefits of milk oligosaccharides compared to fructo- and galacto-oligosaccharides.

Lebrilla's group is one of the pioneers of the quantitative and structural analysis of mammalian milk oligosaccharides[208] which they separate from the lipids and proteins of individual human milk samples and analyse by a combination of microchip LC-MS and MALDI-FTICR-MS.[136] Accurate mass measurements obtained through an orthogonal time-of-flight (o-ToF) MS provides oligosaccharide composition for *ca.* 200 individual molecular species. Comparison of microchip LC-MS profiles from different women revealed inter-individual, lactation phase-dependent and even daily[209] variations in milk oligosaccharide composition. While microchip LC-MS profiling provides routine identification of milk oligosaccharides, tandem MS in combination with exo-glycosidase digestion distinguishes structural isomers.[136]

9.9.10 Milk Lipids

Milk fat is a remarkable source of energy, fat-soluble nutrients and bioactive lipids for mammals. The composition and content of lipids in milk fat vary widely among mammalian species. Milk fat is not only a source of bioactive lipid components; it also serves as an important delivery medium for nutrients, including the fat-soluble vitamins. Bioactive lipids in milk include triacylglycerides, diacylglycerides, saturated and polyunsaturated fatty acids, and phospholipids. Beneficial activities of milk lipids include antimicrobial, anti-inflammatory and immuno-suppressive properties. The major mammalian milk

consumed by humans as a food commodity is that from cows, whose milk fat composition is distinct due to their diet and the presence of a rumen. As a result of these factors, bovine milk fat is lower in polyunsaturated fatty acids and higher in saturated fatty acids than human milk, and the consequences of these differences are being researched.[169]

Odham's group has published an LC-MS/MS study on sphingomyelins as found in an enriched sample of polar lipids from bovine milk.[210] Intact sphingomyelins were separated by normal-phase HPLC and detected by positive mode ESI-MS for structural information. In atmospheric pressure chemical ionisation (APCI), in-source fragmentation of sphingomyelin ions led to the formation of ceramide ions. With the latter as precursors, ions representative of both the long-chain base and the fatty acid parts were detected in APCI-MS/MS *via* collision-induced dissociation (CID). At least 36 protonated molecules of intact sphingomyelin were detected in the bovine milk sample.

Precht *et al*. published comparative studies of isomeric 18 : 1 acids in cow, goat and ewe milk fats by low-temperature high-resolution capillary gas-liquid chromatography, but without deploying mass spectrometry as a detector.[211] The same groups also investigated individual isomeric 18 : 1 acids in cow, goat and ewe milk fats by low-temperature high-resolution capillary gas-liquid chromatography[212] as well as individual *trans*- and *cis*-16 : 1 isomers in the same sources applying the same GC/LC technique.[213]

9.10 Conclusions

Nutrition has a strong influence on immune status, development and decline. Consequently, nutritional modulation of immunity is a major axis in nutrition and health research with the objectives to favourably ''programme'' neonate immunity, maintain immune homeostasis throughout life and reinforce immunity in elderly. Modern immune-modulating nutrition accompanies consumers through their life stages and styles.

An area of immunology and nutrition where mass spectrometry is already a well-established working horse is allergen detection, identification and characterisation. Immune relevant food sources like milk have been extensively investigated by MS in terms of their bioactives complement. A few nutritional interventions have been monitored by MS regarding their immune effects, mainly assessing the PBMC proteome—the latter serving in general as an accessible and relevant immune cell population readily amenable to mass spectrometric proteomics. Intestinal cells have served as a model to study gut immunity by MS means. Moreover, MS is rapidly emerging as the platform complementary to NMR in metabolomic investigations of host–microbe interactions and gut microbiota characterisation.

While mass spectrometry is certainly a most powerful tool to assess immune status and nutritional immune modulation, it is to date largely under-deployed. As the mature and diverse technology platform delivers quantitative,

information-rich data and is highly accurate and sensitive, mass spectrometry in immunology and nutrition means for today and tomorrow:

- discovery of biomarkers for immune status and nutritional intervention;
- mass spectral monitoring of nutritional intervention and bioavailability/bioefficacy studies.

Extending the rather traditional and few molecule-directed bioavailability studies to comprehensive, mass spectrometry-based investigation of metabolism and combining such approaches with MS-rooted proteomics paves the way to proceed from single nutrient bioavailability to multiple-nutrient bioefficacy studies. As mass spectrometry is a central platform to both proteomics and metabolomics, this technology will rapidly expand its role in holistic nutritional biomarker discovery. The nutrition community today largely sticks to traditional proteomic workflows based on 2D gels, but the array of deployed tools will increasingly include stable-isotope and label-free techniques, both enabling a higher throughput.

The complexity and subtlety of improving human health through nutrition requires holistic and sensitive approaches. Due to its versatility, sensitivity, accuracy, information richness and holistic nature, a rapidly expanding business for the application of mass spectrometry to nutrition and health is predicted.

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