

Alternative Protein Sources for Mitigating Post-Weaning Diarrhea in Piglets: Mechanistic Links among Protein Digestibility, Gut Microbiota, and Intestinal Barrier Function and Immune Regulation

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Abstract

Enterotoxigenic Escherichia coli (ETEC) is an important infectious cause of post-weaning diarrhea (PWD) and abrupt feed changes, low feed intake, immature digestive system, microbial dysbiosis, epithelial barrier function and mucosal inflammation have been identified as predisposing factors to and severity of PWD. Dietary protein may play a major role in this, with indigestible protein entering the hindgut potentially stimulating the production of protease fermentation, ammonia, biogenic amines, indoles, phenols and branched chain fatty acids, and antigenic and bioactive protein with effects on the epithelial and immune responses. This review presents a mechanism-based evaluation of the effects of various protein sources on 5 interrelated domains: small intestinal protein digestibility, hindgut fermentation, microbiota composition and metabolic activity, epithelial barrier integrity and immune-inflammatory regulation. Spray-dried plasma is one of the few ingredients reviewed that has relatively widespread evidence of enhancing early post-weaning feed intake and growth performance, with selected controlled studies demonstrating benefits for barrier function, immunity, and diarrhea; however, there are drawbacks with cost and supply, and a biosafety requirement. The effects produced

by products of yeast origin are directed towards microbiota and immunity and vary depending on the type of product used (whole yeast, hydrolysates, cell wall fractions, beta-glucans, mannans or live cultures). There is still limited direct evidence for the protein source of consistent PWD mitigation that is species, processing and inclusion level dependent, though other protein sources such as insect meals, microalgae and novel legumes have potential to diversify protein sources and promote sustainability. No one protein source is optimal to regulate all the pathways that affect PWD. So, in each phase, an appropriate mixture of highly digestible proteins and complementary functional ingredients provides a more biologically sound approach than using only one of the alternative protein sources. Overall, alternative protein sources can help mitigate post weaning diarrhea (PWD) through improvement of protein digestibility, modulation of hindgut fermentation and gut microbiota, support the integrity of the intestinal barrier and help to regulate excessive inflammatory responses; however, the effectiveness is dependent on the protein source, processing conditions, level of inclusion and the physiological and disease state of the piglet.

Keywords

Post-Weaning Diarrhea, Alternative Protein Sources, Protein Digestibility, Proteolytic Fermentation, Gut Microbiota, Intestinal Barrier

1. Introduction

Diarrhea and mortality are one of the most important gastrointestinal diseases in modern pig production which can affect piglet health, growth performance, feed efficiency and survival during the most critical period of development [1]-[3]. When piglets are weaned, they are facing abrupt changes in nutrition, environment, social structure and their digestive, microbial, immune and epithelia systems are not fully mature [4]-[6]. This reduction in feed consumption and digestive enzyme function also causes a reduction in the digestibility of the nutrients and adapts the gut, while the faster remodeling of the gut microbiota also causes loss of gut microbial stability and colonization resistance [2] [3] [7]. These disturbances render the intestine susceptible to disorders and enteric infection. Dietary protein is very important in this regard as piglets at weaning may not be able to digest and absorb all the protein fed to them efficiently [5] [8]. This protein and peptides entering the hindgut undigested serve as substrates for over-fermentation by the proteolytic bacteria and result in the production of ammonia, biogenic amines, indoles, phenols, and branched-chain fatty acids (BCFA) [9] [10]. These metabolites can disrupt the balance of the gut microbiome at higher levels and affect the function of the gut, which may help cause and maintain PWD [2] [11].

Soybean meal is the predominant plant-protein ingredient used in pig diets because of its relatively high protein content, broad availability, and generally favorable amino acid profile. However, its use at high inclusion levels during the early

post-weaning period may be constrained by residual anti-nutritional factors and antigenic proteins, particularly trypsin inhibitors, glycinin, and β -conglycinin [12]. These compounds can interfere with proteolytic digestion, provoke hypersensitivity or inflammatory responses, impair intestinal morphology and barrier function, and ultimately reduce nutrient utilization in newly weaned piglets [13] [14]. Accordingly, the suitability of a protein ingredient for newly weaned piglets should be assessed beyond its crude protein content and amino acid composition, taking into account ileal amino acid digestibility, processing-related changes, residual antinutritional factors, effects on hindgut fermentation, and functional effects on intestinal physiology. At the same time, restrictions on antibiotic growth promoters and the phase-out of pharmacological zinc oxide in several production systems have intensified the search for nutritional interventions capable of maintaining intestinal health and growth performance after weaning [1] [15]. These developments have increased interest in alternative protein sources that may provide both nutritional value and functional benefits for post-weaning intestinal health.

In recent years, however, the use of new piglet nutrition has evolved beyond simply replacing conventional protein ingredients, and focused on the development of protein strategies optimized for improved protein functionality during the post-weaning transition, particularly in the gastrointestinal tract [16]. A desirable protein source should not only supply the piglets with a balanced mixture of digestible amino acids but also minimize the amount of undigested protein entering the hindgut, minimize the presence of antinutrients, maintain the integrity of the gut, maintain a stable and beneficial microbial ecosystem and regulate excessive inflammatory response [17]. These traits are now known to play a major role in gut health post-weaning as they affect multiple other biological functions that play a role in the development of PWD [18] [19]. Thus, dietary protein ingredients should be assessed in relation to nutritional quality and modulation of host-microbiota interface and intestinal homeostasis as well, considering both nutritional and functional aspects [1] [4] [6].

Dietary protein digestibility is an important factor that is affecting the post-weaning intestinal health since it affects the availability of amino acids to the piglet as well as the amount of undigested nitrogenous substrates entering the distal intestine. Therefore, protein source and quality may impact proteolytic fermentation in the hindgut, microbial community structure, intestinal barrier function and inflammatory responses. Therefore, protein selection is one of the possible nutritional interventions to minimize PWD risk [1] [2]. These biological functions are tightly interlinked and dietary protein affects gastrointestinal homeostasis via concerted actions on digestion, the gut microbiota, epithelial barrier function, and immune reactions [1] [2] [9].

Various alternative protein sources, including fermented soybean meal, spray-dried plasma, yeast-derived products, insect proteins and new ingredients (e.g., microalgae and novel legumes) have been studied for their ability to reduce post-

weaning diarrhea [20]. But these positive effects are not fully coalesced throughout the literature. The majority of studies have assessed individual protein sources in isolation and/or only one biological outcome, such as growth performance, nutrient digestibility, and microbial composition, without considering these data in the context of the overall biological systems underlying intestinal homeostasis and PWD pathogenesis [18] [21] [22]. It is also challenging to determine the main protein source of protective effects, and to systematically compare the functional roles of different protein sources. This review aims to build an overall mechanistic overview of dietary protein sources and their association with intestinal health after weaning, by combining information on protein digestibility, large intestine fermentation, microbiota modulation, intestinal barrier integrity and immune regulation. This review provides a biological insight to understand the functional role of different protein sources to devise nutritional strategies to prevent digestive disorders after weaning weaned piglets.

Selection and Interpretation of Literature

This is a narrative and mechanism-oriented review and not a systematic review or meta-analysis. The four electronic databases are Google Scholar, PubMed, Web of Science and Scopus to conduct literature search. All publications from 2000 to 2026 were taken into account and particular focus was given to publications in the 2020-2026 period. Literature search targeted studies that examined alternative protein sources in weaned piglets and their association with nutrient digestibility, gut fermentation, microbial composition and function, epithelial barrier integrity, immune response and clinical diarrhea.

Feed trials and challenge experiments with piglets were given preference for efficacy evaluation of ingredients in primary feeding trials, while review articles and non-piglet experimental studies were only considered when they provided relevant mechanistic background. Evidence interpretation included consideration of study population, diet formulation, ingredient processing, and inclusion level, challenge conditions, outcome definitions, and directness of evidence. This was a narrative review so conclusions relied on consistency and biological relevance of available evidence and not quantitative meta-analysis.

2. Pathophysiology of Post-Weaning Diarrhea

Post-weaning diarrhea (PWD) is a multi-factorial syndrome resulting from a combination of dietary changes, feed intake, changes in gut maturation, microbial balance and pathogen exposure that exceed the piglet's ability to maintain gut homeostasis. Experimental evidence shows that there are rapid changes in several functions of the villus and crypt architecture, brush-border enzyme activity, epithelial transport and permeability, as well as epithelial cytokine expression after weaning, that diminish digestive and absorptive efficiency and increase the vulnerability of the epithelium [23] [24]. The distal intestine is exposed to higher concentrations of incompletely digested nutrients, such as nutritional protein, un-

der these conditions, which can modify the availability of substrates, the microbial biochemistry and the physicochemical parameters of the lumen. The results of controlled challenge studies indicate that low crude protein intake yields firmer feces and results in fewer PWD cases and indices of proteolytic fermentation, while high protein or high casein diets exacerbate diarrhea and alter gut microbiota. Therefore, *enterotoxigenic Escherichia coli* is considered to only be part of a wider range of interlinked mechanisms contributing to the development of PWD, including digestive insufficiency, microbial dysbiosis, epithelial barrier dysfunction and mucosal inflammation.

The proteins (from feed or endogenous source) that escape digestion in the small intestine are degraded in the hind gut by microbial proteases and peptidases, then the amino acids and peptides produced are used as building blocks for microbial biomass or are fermented via deamination, decarboxylation and other pathways. These reactions produce a heterogeneous set of metabolites such as ammonia, branched chain fatty acids, biogenic amines, hydrogen sulfide, indoles, phenols, skatole and p-cresol [25] [26]. The physiological effects of compounds are metabolite-, concentration-, and site-dependent; although some amino acid-derived products may exert signaling or metabolic functions at physiological concentrations, their excessive accumulation can disturb colonocyte energy metabolism, epithelial turnover, barrier integrity, and intestinal water and electrolyte transport [27]. High dietary protein intake generally increases the intestinal concentrations of several protein-derived metabolites and has been associated with altered microbial activity, mucosal inflammatory responses, reduced fecal consistency, and a greater risk of PWD [25]. However, proteolytic and saccharolytic fermentation should not be viewed as strictly opposing processes because their relative contributions depend strongly on the availability of fermentable carbohydrates and the carbon-to-nitrogen ratio of the digesta. Increasing fermentable carbohydrate availability can redirect nitrogen toward microbial protein synthesis and reduce several potentially harmful protein-derived metabolites, although ammonia alone is an insufficient indicator of the overall extent of protein fermentation [10]. Thus, the pathological significance of hindgut protein fermentation is determined less by fermentation itself than by the quantity, composition, and intestinal accumulation of the resulting metabolites. Experimental evidence shows that the two pathways may occur simultaneously and that their relationship is strongly influenced by digesta composition and the carbon-to-nitrogen ratio [10].

2.1. Microbiota Dysbiosis and Loss of Stability

During weaning, the intestinal microbiota also goes through a rapid ecological succession when milk nutrients are replaced by a more complex solid feed containing plant proteins, starch and non-digestible carbohydrates. This physiological transition should not in itself be considered as dysbiosis and only with acute change in the diet, a decrease in feed intake, environmental stress, exposure to a pathogen or immature host defense is it considered as dysbiosis [28] [29].

Importantly, there is no single universal microbial signature of PWD as microbiota response depends on age, diet, housing conditions, antimicrobial exposure, genotype and intestinal sampling site. However, piglets that are susceptible to or affected by diarrhea often have impaired microbial stability and fermentation function, and an increase in facultative anaerobic bacteria and pathobionts in the gut, especially of *Enterobacteriaceae* such as *enterotoxigenic Escherichia coli* [2]. Ecological opportunities for the attachment, proliferation, and enterotoxin production of pathogens increase, thereby weakening colonization resistance. Microbial dysbiosis and intestinal dysfunction could perpetuate each other during the post-weaning period: dysbiosis and changes in metabolite production can lead to intestinal barrier failure and inflammatory response of the mucosa, and intestinal barrier failure during weaning and inflammatory response of the mucosa can affect the stability of the intestinal microbial community [2] [30]. However, experimental data indicate that FMT early in life decreases diarrhea, enhances expression of tight junctions and reduces intestinal inflammation, suggesting that the microbiota is not just a by-product of the disease [30].

The decrease in feed intake and decrease in luminal stimulation during weaning contribute to a shortening of the villi, remodeling of the crypts, and decreased absorption [31]. However, there are different outcomes, namely, intestinal morphology and functional barrier integrity: recovery of villus architecture does not necessarily indicate that epithelial permeability or tight-junction regulation has normalized [32]. This enhanced permeability can allow the mucosal immune cells to come into contact with the dietary antigens and microbial products, which leads to cytokine and MAPK-mediated responses that may further impair epithelial transport and barrier function [23] [32] [33].

2.2. Intestinal Barrier Dysfunction and Mucosal Inflammation

The intestinal epithelial barrier is a dynamic defense system that includes the mucous barrier, epithelial continuous sheet, intercellular junctional complexes, antimicrobial secretions and underlying mucosal immune system components. In the immediate post-weaning period, lower feed intake, sharp dietary changes and lower luminal stimulation are all important factors that cause villus shortening, crypt remodeling, lower epithelial renewal and decreased absorptive capacity [31] [34]. In addition to these structural changes, transepithelial electrical resistance decreases, paracellular permeability increases, ion transport changes and tight junction proteins (e.g., zonula occludens-1, occluding, and claudins) expression and localization are altered. Weaning is therefore correlated with a rise in intestinal expression of pro-inflammatory cytokines, including interleukin 1 beta (IL-1 β), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF- α), as well as activation of stress-responsive signaling pathways, including nuclear factor kappa B (NF κ B) and mitogen-activated protein kinases (MAPKs) [23] [32]. Barrier dysfunction and mucosal inflammation are in a vicious cycle: with barrier dysfunction leading to immune activation and vice versa, leading to increased suscepti-

bility of weaned piglets to enteric infection and diarrhea.

2.3. *ETEC* Colonization and Enterotoxin-Mediated Secretory Diarrhea

Enterotoxigenic Escherichia coli (*ETEC*) is an important infectious factor in the development of PWD, and requires successful colonization of the intestine and susceptibility of the host for disease development [35] [36]. In the case of porcine *ETEC*, fimbriae containing either F4 or F18 are frequently expressed, which bind to epithelial cells of the small intestine, and are followed by the production of heat-labile (LT) and/or heat-stable (STa and STb) enterotoxins [37]-[39]. These toxins induce secretory diarrhea through the cAMP-, cGMP- and calcium-associated signaling pathways, which disrupt intestinal ion transport leading to secretion of electrolytes and water [37] [40]. *ETEC* infection also may disrupt the integrity of the tight junctions and induce inflammation of the mucosa, which would further exacerbate the intestinal dysfunction observed during weaning [35] [41]. Thus, the diarrhea is not just due to gross mucosal destruction but mainly secretory. However, it may also affect tight-junction organization, affect epithelial renewal, induce oxidative stress, and induce inflammatory responses of the mucosa, which may link the enterotoxin-mediated secretion with the underlying microbial, epithelial, and immune disturbances already present as a result of the weaning stress [35] [42].

2.4. Integrated Pathophysiological Cycle

The pathogenesis of post-weaning diarrhea is better conceptualized as a self-reinforcing biological network than as a simple linear sequence of events. After weaning, reduced feed intake, immature digestive capacity, and impaired nutrient absorption increase the delivery of undigested substrates to the distal intestine [4]. These substrates alter microbial community structure and fermentation activity, potentially promoting the expansion of enteric pathobionts and the accumulation of metabolites that impair epithelial function [2] [5]. The resulting inflammatory response further disrupts tight-junction organization, epithelial renewal, nutrient transport, and intestinal water and electrolyte homeostasis, thereby intensifying diarrhea and growth impairment [34]. The severity of clinical PWD depends on the interaction between pathogen exposure and the piglet's nutritional, microbial, epithelial, and immunological status [42]. Consideration then must be given to gastrointestinal dysfunction that may be further enhanced by increased intestinal transit, nutrient loss, decreased appetite and delayed recovery of the gut lining. This integrated model accounts for why single target interventions on a single pathological process frequently yield variable results and why nutritional strategies that target multiple processes and/or have multiple functions might be more predictable [1].

It is now recognized that post-weaning diarrhea is a self-reinforcing disorder, made up of a combination of nutritional, microbial, epithelial, immunological and

infectious events. The sudden removal from the sow, reduced feed consumption, change of feed and immaturity of gut function all impair the ability to hydrolyze and absorb nutrients, allowing more undigested protein (e.g., dietary protein and endogenous protein) to pass down into the distal gut [5] [34]. This change in substrate availability may further enhance the proteolytic fermentation, change the substrate-metabolite profile in the lumen and disrupt the structure of the microbial community, which consequently decreases colonization resistance and increases the opportunity for enteric pathobionts to proliferate [2].

Post-weaning diarrhea is a self-exacerbating condition where nutrition, microorganisms, epithelium, immune system and infectious diseases play a role [18] [43]. The decreased feed intake and digestive capacity of the animal around weaning lead to more undigested protein reaching the distal intestine and thus to more proteolytic fermentation and microbial instability [5] [18] [44]. This disruption of the barrier and increase in intestinal permeability can in turn increase the exposure of the mucosa to microbial products and dietary antigens and augment inflammatory responses that can further compromise epithelial integrity, nutrient transport, and intestinal recovery [2] [18] [45] [46]. Colonization by fimbriae, plus fluid and electrolyte secretion in response to *ETEC* can lead to this cycle [43] [47] [48]. Therefore, nutritional strategies to enhance performance should focus on several interconnected processes, such as protein digestibility, microbial stability, hindgut protein fermentation, maintenance of integrity of the epithelia and regulation of inflammation [45] [49]. Thus, evaluation of the ability of alternative protein sources to impact at one or more of these linked nodes should be done in addition to crude protein content and amino acid profiles.

The dietary, environmental, and physiological stressors involved in weaning lead to decreased digested efficiency, decreased intake of feed, dysbiosis of intestinal microbiota and impaired barrier integrity of the gut epithelial cells [23] [32]. Post-weaning microbial dysbiosis could include a decrease in microbial ecological stability, fermentation and colonization resistance, and a variable microbial profile between individual piglets and production environment [50]. The translocation of microbial products and dietary antigens that occurs due to villus atrophy, disruption of tight-junction proteins, and increased intestinal permeability is responsible for the activation of NF- κ B- and MAPK-dependent inflammatory pathways. *ETEC* with F4 or F18 fimbriae bind to the small-intestinal epithelium and secrete heat-labile and heat-stable enterotoxins that disrupt cyclic nucleotide signaling, leading to chloride/bicarbonate secretion, decreased sodium absorption and secretory diarrhea [51]. These processes build up a vicious circle of sub-par digestion, overload of protein in the hindgut, proteolytic fermentation, microbial dysbiosis, barrier failure, inflammation, decreased feed intake and delayed intestinal recovery. Nutritional interventions can break the cycle by enhancing protein digestibility, minimizing the presence of antinutritional factors, limiting undigested protein in the hindgut, maintaining a stable gut microflora, maintaining integrity of the intestinal epithelium and reducing excessive inflammation [52].

Abbreviations used in the integrated model: BCFA, branched-chain fatty acids; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; ETEC, *enterotoxigenic Escherichia coli*; IL, interleukin; LT, heat-labile *enterotoxin*; MAPK, mitogen-activated protein kinase; NF- κ B, nuclear factor kappa B; PWD, post-weaning diarrhea; STa and STb, heat-stable enterotoxins A and B; TNF- α , tumor necrosis factor alpha; ZO-1, zonula occludens-1.

3. Mechanistic Framework Linking Dietary Protein Sources to Gut Health

The effect of dietary protein source on post-weaning intestinal health is determined by more than just its nutritional value; it requires a series of interrelated biological pathways. These processes include small intestinal protein digestibility, hind gut fermentation, microbiota composition and metabolic activity, epithelial barrier integrity and immune inflammatory regulation [2]. All these biological processes are not independent but interact with each other to decide the intestinal homeostasis and susceptibility to postweaning diarrhea (PWD). The integrated pathophysiological events underlying the pathogenesis of postweaning diarrhea in piglets are summarized in **Figure 1** and the key nutritional intervention points are indicated at which alternative protein sources might have an effect on the complex chain of events.

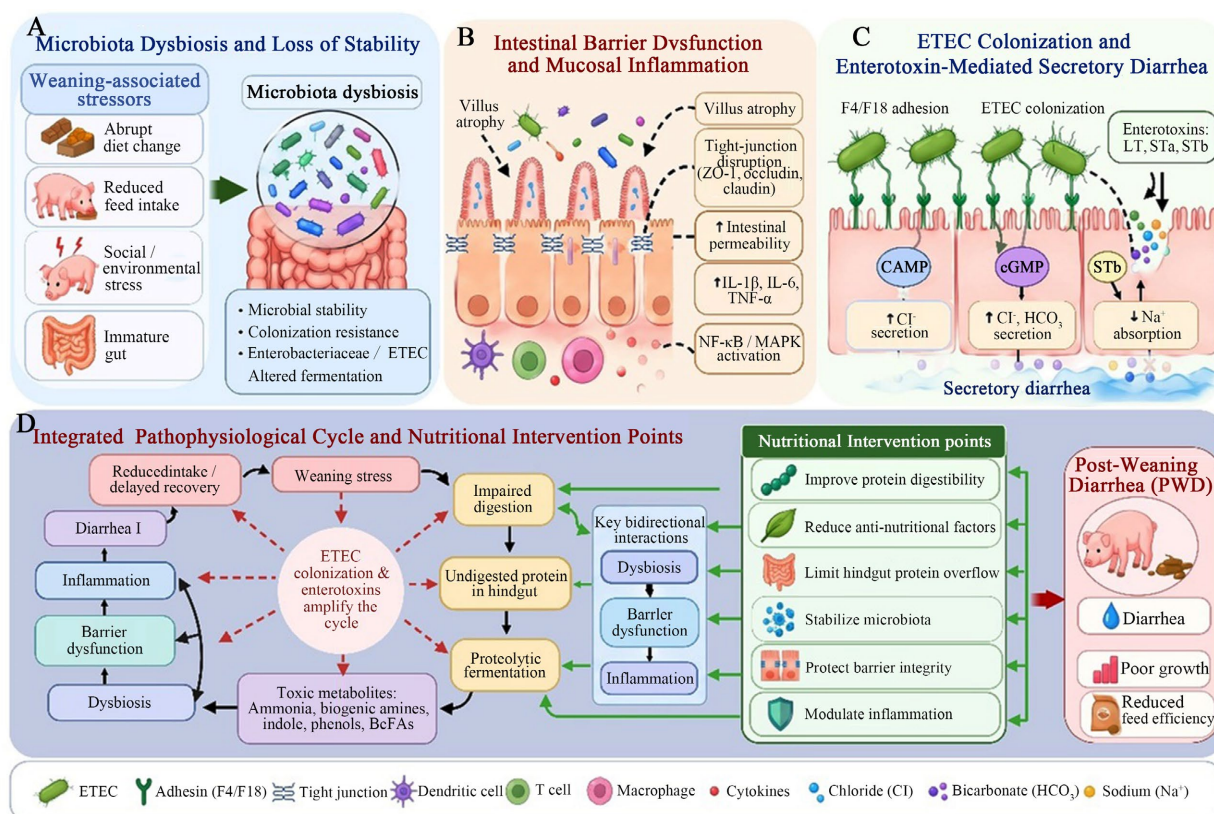


Figure 1. Integrated pathophysiological mechanisms in post-weaning diarrhea in piglets and main nutrition intervention points.

The pathophysiological network summarized in **Figure 1** serves as a basis for assessing alternative protein sources, based not only on nutrient composition, but also on biological function. In this context, the impact of dietary proteins on gut health after weaning can be classified into five interconnected mechanistic domains: small-intestinal protein digestibility, hindgut fermentation, composition and activity of the intestinal microbiota, integrity of the epithelial barrier and immune-inflammatory modulation [2].

Table 1 summarizes the major features and biologic roles of the five mechanistic domains. These domains are hierarchical and not autonomous. Digestion of dietary proteins in the small intestine affects the amount and biochemical composition of dietary proteins reaching the large intestine, whilst the products of microbial fermentation will affect the ecology, metabolism of epithelial cells, permeability of epithelia, and immune signaling [8] [9]. On the other hand, if the intestine becomes inflamed and damaged, the activity of digestive enzymes and the absorptive capacity may be lowered, causing an increase in overflow of nutrients and stimulating microbial fermentation.

A linear relationship between crude protein content, amino acid balance/clearly digestibility and incidence of diarrhea was not observed in this reciprocal organization. The mechanism of action of the protein ingredient may be direct, while that of another may be mediated by antimicrobial peptides, immunoglobulins, bioactive polysaccharides or other functional components [2] [5]. Alternative proteins should then be evaluated based on the “where”, “how much” and “how consistently” they make an impact, and if an impact on a biomarker is followed by a direct clinical impact, within the five mechanistic domains [2] [5].

Table 1. Mechanistic domains of the effects of dietary protein sources on post-weaning diarrhea in piglets.

Mechanistic domain	Protein-source characteristics involved	Principal biological processes	Recommended biomarkers and indicators	Expected implication for PWD	Important interpretive limitation	References
Small-intestinal protein digestibility	Protein structure and solubility; peptide size; amino acid composition; processing severity; protease inhibitors; antigenic proteins; fiber content	Gastric, pancreatic, and brush-border hydrolysis; peptide and amino acid absorption; endogenous amino acid losses	Apparent ileal digestibility; standardized ileal digestibility; ileal undigested nitrogen; pancreatic and brush-border enzyme activities; plasma or blood urea nitrogen	May reduce the amount of undigested protein reaching the hindgut and limit excessive proteolytic fermentation	High digestibility does not necessarily indicate reduced antigenicity, improved barrier protection, or immunomodulatory activity	[52]-[54]
Hindgut protein fermentation	Ileal protein escape; amino acid composition; endogenous protein secretions; fermentable carbohydrate supply; digesta carbon-to-nitrogen ratio	Microbial proteolysis, deamination, decarboxylation, and aromatic amino acid catabolism	Ammonia; BCFA; biogenic amines; indoles; phenols; skatole; p-cresol; hydrogen sulfide; SCFA; digesta pH	May reduce excessive accumulation of protein-derived metabolites and contribute to a more balanced hindgut fermentation environment	Ammonia alone is insufficient to characterize protein fermentation; metabolite effects depend on concentration, intestinal site, and interactions with carbohydrate fermentation	

Continued

Microbiota composition and metabolic function	Substrate availability; protein fermentability; peptide profile; residual carbohydrates; organic acids; fermentation-derived compounds; microbial or yeast cell-wall components	Microbial community restructuring; pathogen competition; colonization resistance; microbial metabolite production; functional pathway regulation	Alpha and beta diversity; <i>Enterobacteriaceae</i> ; <i>Escherichia-Shigella</i> ; <i>Lactobacillus</i> , <i>Prevotella</i> ; metagenomic functions; SCFAs; ammonia; indoles; phenols; pathogen load	May support microbial stability, enhance colonization resistance, and reduce ecological conditions favorable for <i>ETEC</i> proliferation	Changes in individual bacterial taxa should not be classified as beneficial or harmful without corresponding functional, metabolic, and host-response data Improvements in morphology or tight-junction-related markers do not necessarily indicate complete restoration of epithelial barrier function unless supported by functional permeability measurements Immune effects may be direct or secondary to changes in digestibility, microbiota, fermentation metabolites, or intestinal permeability	[56] [57]
Intestinal barrier integrity	Bioactive peptides; immunoglobulins; growth factors; antioxidants; antigenic proteins; microbial metabolites; fermentation products	Epithelial renewal; villus maintenance; mucus production; tight-junction assembly; paracellular permeability; epithelial repair	Villus height; crypt depth; villus-height-to-crypt-depth ratio; ZO-1; occludin; claudins; mucins; transepithelial electrical resistance; FITC-dextran permeability; serum diamine oxidase; D-lactate	May reduce epithelial leakage and antigen translocation and contribute to reduced susceptibility to inflammatory and secretory diarrhea		[32] [52] [58]
Immune and inflammatory regulation	Immunoglobulins; β -glucans; mannans; bioactive peptides; growth factors; antigenic proteins; antioxidant compounds	Pattern-recognition receptor activation; NF- κ B and MAPK signaling; cytokine production; mucosal antibody responses; immune-cell recruitment and regulation	IL-1 β IL-6; IL-8; IL-10; TNF- α ; interferon- γ ; NF- κ B activation; MAPK phosphorylation; secretory IgA; IgG; IgM; inflammatory-cell infiltration	May regulate excessive mucosal inflammation, support immune defense, and contribute to epithelial recovery		[52] [58] [59]

Note: BCFA, branched-chain fatty acid; *ETEC*, enterotoxigenic *Escherichia coli*; FITC-dextran, fluorescein isothiocyanate-dextran; MAPK, mitogen-activated protein kinase; NF- κ B, nuclear factor kappa B; PWD, post-weaning diarrhea; SCFA, short-chain fatty acid; ZO-1, zonula occludens-1. The mechanistic domains are biologically interconnected and should not be interpreted as independent pathways. Improvements in individual biomarkers should not be considered direct evidence of PWD prevention unless supported by integrated clinical, microbial, metabolic, epithelial, and immunological outcomes.

3.1. The Small-Intestinal Protein Digestibility Is an Upstream Regulatory Node

Intestinal digestibility is the first step and the first mechanism for dietary protein sources to impact post-weaning gut health. The digestibility of crude protein depends not only on its content, but also on the structure or solubility of proteins, amino acid composition, endogenous amino acid losses, the severity of the processing, and other nutrient inhibitors such as fiber, protease inhibitors, antigenic proteins, etc. [8]. Total-tract digestibility is less useful in amino acid evaluation than ileal digestibility because nitrogenous compounds may be altered by microbial metabolism after the ileum and this will not reflect the quantity of the amino acids of the dietary protein that are available for absorption by the host [60] [61]. The apparent ileal digestibility (AID) is the digestibility of dietary and endogenous amino acids in the digesta before they reach the terminal ileum, while the standardized ileal digestibility (SID) is the digestibility of dietary amino acids after the terminal ileum corrected for basal endogenous amino acid losses, which is more

appropriate for comparing individual feed ingredients and for use in the formulation of mixed diets.

In ileal-cannulated weaned pigs, two fermented soybean meal formulations were able to improve the apparent and standard ileal digestibility of crude protein and amino acids and reduced the major antinutritional factors found in soybean meal [52]. Other comparisons have shown different results with one study finding good apparent ileal digestibility values for fermented soybean meal [62] and one more recent finding similar values for amino acid digestibility as compared to conventional soybean meal [63].

Higher ileal protein and amino acid digestibility may reduce the amount of undigested nitrogenous substrate reaching the hindgut and thereby limit the potential for excessive proteolytic fermentation [8]. However, digestibility alone does not establish gut-health efficacy, because protein ingredients may differ in antigenicity, bioactive components, microbial fermentability, and effects on epithelial barrier and immune function [52]. Ileal digestibility should therefore be interpreted together with fermentation, barrier, immune, and clinical outcomes when evaluating alternative protein sources for PWD mitigation.

3.2. Regulation of Hindgut Protein Fermentation

The remaining dietary and endogenous proteins in hind gut are hydrolyzed by microbial proteases/peptidases to peptides and amino acids which are then metabolized by deamination, decarboxylation and catabolism of aromatic amino acids. These reactions result in the production of ammonia, branched-chain fatty acids, biogenic amines, indoles, phenols, skatole, hydrogen sulfide and p-cresol, some of which can have negative effects on the metabolism and integrity of the epithelium when produced in too large quantities [9] [25]. High protein content in the diet and the use of poorly digestible protein ingredients tend to lead to higher N-substrate availability and thus to a more proteolytic fermentation profile, which can negatively affect the microbial composition, fecal consistency, epithelial metabolism and integrity and increases the risk of diarrhea in weaned piglets [11] [56].

Highly digestible sources of protein, or a proper reduction in dietary protein, can on the other hand reduce the amount of fermentable nitrogen reaching the hindgut, and the production of some of the potentially harmful metabolites [55] [64], while retaining the level of essential amino acids. Fermentable carbohydrates are also involved in this process, as they provide microbial energy and stimulate the incorporation of nitrogen into microbial biomass, thus shifting metabolism towards saccharolytic fermentation and increasing the production of short-chain fatty acids, especially acetate, propionate and butyrate [10]. With this in mind, alternative protein sources must also be evaluated for their impact on hindgut substrate balance and the profile of microbial metabolites if effective control of PWD is to be achieved in order to minimize the amount of the harmful protein-derived products whilst maintaining the beneficial microbial fermentation.

3.3. Modulation of Microbiota Composition and Metabolic Function

Protein source and processing method in the diet influence the gut microbial composition and fermentation in weaned piglets, but this is ingredient and context specific. In piglets challenged with *ETEC*, fermented soybean meal affected cecal microbiota, with a reduction in the abundance of Proteobacteria, *Actinobacillus* and *Escherichia-Shigella* bacteria, and with improved growth, fecal consistency and inflammatory profile [57]. Likewise, fermented corn-soybean meal boosted the levels of *Lactobacilli*, *faecalibacteria*, and butyrate in the colon, and total replacement of the maize meal prevented diarrhea in the rats [65]. Thus, microbiota-related effects of alimentary protein sources should be considered in conjunction with microbial metabolites and host-health outcomes and not based on changes in individual microbiota taxa. Comparisons between fermented soybean meal, fish meal and a blend of the two also show that different protein sources can differently modify the ileal and colonic bacterial community, confirming that protein source affects microbiota responses and these responses are intestinal site specific [66].

3.4. Maintenance of Intestinal Barrier Integrity

The intestinal epithelial barrier is a key downstream point of convergence where dietary protein sources can contribute to either exacerbation or amelioration of PWD [2] [44]. Protein ingredients can affect barrier integrity indirectly by altering the quantity of undigested protein and antigenic compounds that reach the distal intestine and, subsequently, the production of microbial metabolites that are able to harm epithelial cells [11] [67]. They can also exert direct effect via the release of immunoglobulin, bioactive peptides, growth factors, antioxidants and fermentation-derived compounds that affect the proliferation of epithelium, *mucosogenesis*, tight-junction assembly and mucosal repair [15] [68]. The coordinated regulation of villus architecture, epithelial turnover, goblet cell activity, mucin production, and the expression of junctional proteins (e.g., zonula occludens-1, *occludin*, and *claudins*) is essential for maintenance of a functional barrier [69]. Therefore, villus height and villus-height to crypt-depth ratio should not be used alone as evidence of barrier restitution as structural restitution can occur before transepithelial resistance and paracellular permeability become normalized [32]. Thus, barrier assessment should include both histological and molecular markers and functional markers such as villus morphology, markers of mucin expression, markers of tight-junction abundance and localization, markers of transepithelial electrical-resistance, markers of macromolecular flux, serum D-lactate or diamine oxidase, and urine or serum markers of permeability.

Experimentally, it has been shown that different protein sources have different mechanisms of action in support of barrier integrity. Beyond its superior digestibility, SDPP has bioactive and immunological properties which can be beneficial in newly weaned pigs by promoting intestinal barrier function and limiting mu-

cosal inflammation and diarrhea [58]. Soaked soy proteins could exert their effect both by decreasing antigenicity and by producing functional peptides. Fermented soybean meal and its peptide fractions have been linked to upregulation of mucin and tight-junction protein expression, a decrease in inflammatory and permeability markers in the blood, and an increase in resistance of piglets' and porcine intestinal epithelial cells to intestinal permeability [70]. Like that, fermented corn-soybean meal decreased diarrhea, and improved intestinal barrier associated outcomes and microbial fermentation in weaned piglets, demonstrating the interaction between substrate processing, microbiota activity and protection of the intestinal barrier [65]. These results suggest that barrier protection can be achieved by multiple non-equivalent mechanisms: reducing exposure to soybean antigens and toxic fermentation products, providing bioactive molecules that strengthen epithelial barrier, facilitating the renewal of mucous membrane and epithelium, and inhibiting inflammation that could destabilize barrier. Alternative protein sources should thus be considered barrier-supportive if morphology and/or expression of tight-junctions improve and if possible, if morbidity due to diarrhea decreases.

3.5. Immune and Inflammatory Regulation

One of the most important downstream pathways by which dietary protein sources affect intestinal recovery and susceptibility to PWD is immune-inflammatory regulation [5]. Microbial dysbiosis, epithelial leakage, soybean derived antigens, and *ETEC* associated molecular signals can be recognized by pattern-recognition receptors and trigger downstream NF- κ B and MAPK pathways and consequently increase inflammatory mediator production, including IL-1 β , IL-6, IL-8, TNF- α and interferon- γ [32] [71]. While transient immune activation is indispensable for the defense against pathogens and the repair of tissues, an exaggerated or protracted immune activation leads to increased permeability of the epithelia, a change in intestinal secretion and absorption, and a shift in amino acids and energy from growth to immune function. Alternative protein sources can indirectly regulate this response by making the protein more digestible, by limiting exposure to antigenic proteins, by reducing toxic fermentation products and by stabilizing the composition of the microbiota, or directly by the presence of active components of the protein such as immunoglobulins, bioactive peptides, β -glucans, mannans, growth factors, or other functional components [71]. An example of a direct immunological activity could be the effects of spray dried porcine plasma on intestinal levels of TNF- α and interferon- γ , inflammatory-cell accumulation, and the integrity of the epithelia, and on fecal score in pigs just after weaning, as dietary inclusion of plasma resulted in clear coordinated effects on this mucosal immunity and barrier function that could not only be a purely nutritional response [58].

Fermented soybean meal may have a dual mechanism of reduced antigenicity and the production of bioactive compounds via the fermentation process; in weaned pigs, fermented soybean meal produced a higher serum IL-4, and higher

levels of immunoglobulin A, G, and M, while decreasing the levels of IL-2 and IL-6, although the small experimental sample size and systemic rather than mucosal markers preclude direct inferences about protection against PWD [52]. A purified glucan from *Saccharomyces cerevisiae* and related yeasts appeared to have an effect on downregulating a number of pro- and anti-inflammatory cytokine transcripts in pig intestinal tissue and also reducing the levels of *Enterobacteriaceae*, although caution needs to be exercised when considering the immune equivalence of all yeast products owing to the different glucan structures, solubility, sources and dosages [59]. Therefore, the ideal goal is not to simply suppress the immune response but to restore immune homeostasis at the mucosal level, so that the immune response is adequate to control pathogens, and inflammation is controlled, preventing excessive inflammation that would worsen barrier disruption and diarrhea.

3.6. Integration of the Five Mechanistic Pathways

Small intestinal digestibility, hind gut fermentation, microbiota modulation, epithelial barrier integrity and immune-inflammatory regulation can be studied individually, but are believed to be an overwhelming connected biological network [2]. It is proposed that alternative protein sources interact with the major biological processes related to intestinal homeostasis, as summarized in **Figure 2**. The figure shows the relationship between protein digestibility, hindgut fermentation, regulation of the gut microbiota, integrity of the gastrointestinal barrier and immune-inflammatory responses. It also demonstrates that the impact of alternative proteins is not only through one pathway but by coordinated activities at nutritional, microbial, epithelial and immunological levels. The small intestinal protein utilization is the principal upstream control point which regulates nitrogenous matter flow and composition to the hind gut. However, digestibility is not the only factor which is influencing the gut-health response [72] [73]. In piglets fed a high protein diet, an increase in ammonia, branched-chain fatty acids, biogenic amines, indoles and phenols in the ileum due to undigested protein could affect the metabolism of the epithelium and cause diarrhea, which has been observed [56] [74]. When barrier function is damaged, it leads to increased exposure of the mucosal immune cells to microbes and food molecules, which further damages tight-junction structure, digestive function and nutrient uptake, causing more overflow of substrates and perpetuating the cycle [32]. There are several points in this network where alternative proteins can be used to disrupt it. In general, fermented soybean meal exerts its effects mainly upstream, by decreasing the presence of antinutritional factors and increase the digestion of nutrients; however, this can include the antioxidant activities, intestinal morphology and immune responses [52]. In addition to a high nutritional value, spray dried porcine plasma (SDPP) also harbours immunoglobulins and other bioactive ingredients that directly affect the level of inflammation at the mucosa, the permeability of the mucosa and the severity of the diarrhea [58]. Thus, evaluation of the efficacy of an

alternative protein source should be performed in a hierarchically structured evidence framework that includes: 1) primary nutritional effects such as ileal digestibility and reduction in antinutritional factors; 2) intermediate mechanistic responses such as fermentation metabolites, microbiota function, epithelial permeability and immune signaling; and 3) clinically relevant outcomes such as incidence of diarrhea, fecal consistency, feed intake, growth rate and feed efficiency. This approach helps to prevent that efficacy is only credited to a single biomarker and helps to give a solid foundation to compare different protein sources later in this section. Alternate protein sources can affect various nodes in this biological network, presenting numerous opportunities to prevent intestinal dysfunction and promote post-weaning health as shown in **Figure 2**.

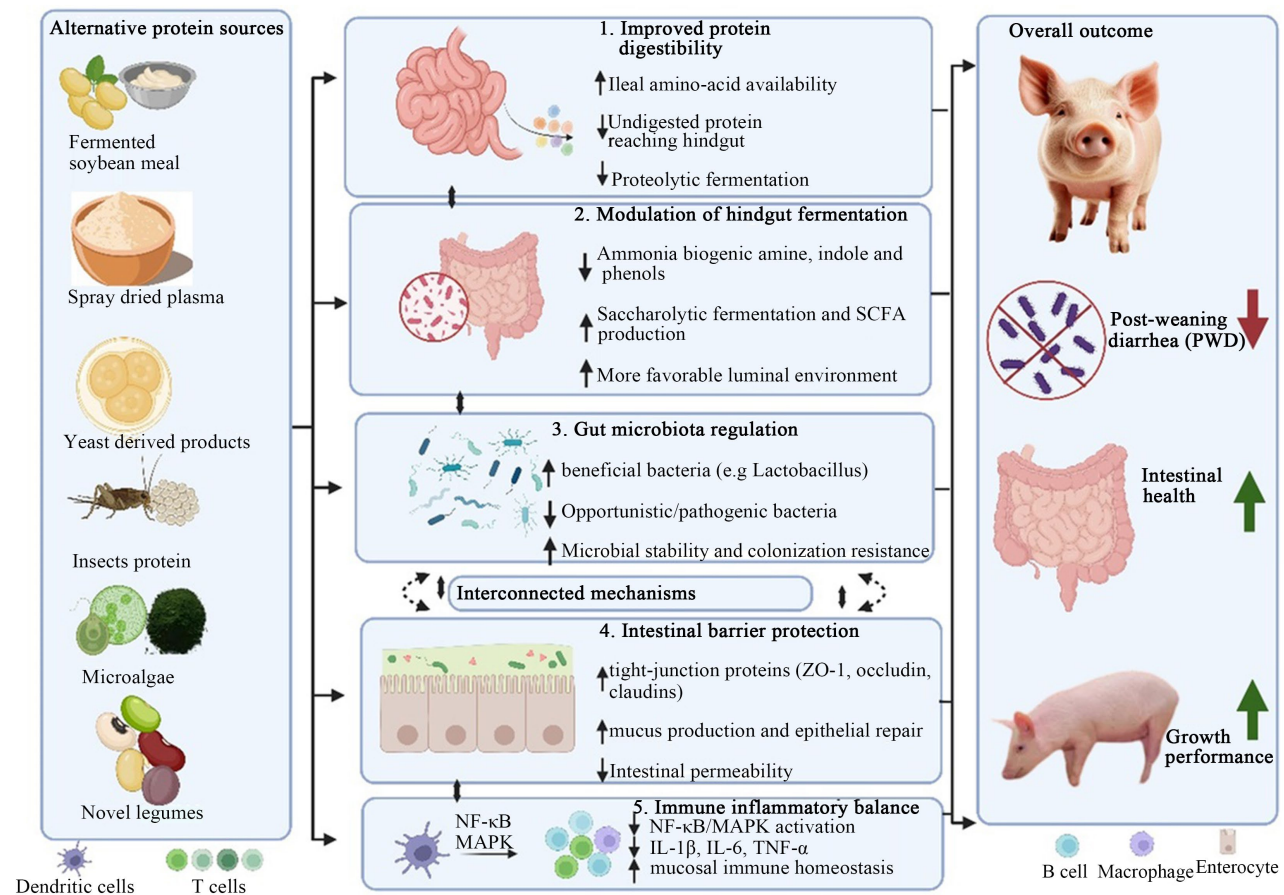


Figure 2. Proposed mechanisms by which alternative protein sources modulate intestinal digestion, microbiota, epithelial barrier integrity, and immune responses to alleviate post-weaning diarrhea in piglets.

4. Comparative Evaluation of Alternative Protein Sources for PWD Mitigation

Now that the five mechanistic domains of how dietary protein can affect post-weaning gut health are known, alternative protein sources can be evaluated based on their predominant biological action(s), strength of evidence, and practicality, as well as crude protein concentration. The ileal digestibility, antinutritional-fac-

tor burden, bioactive-component composition, fermentability, and impact on host-microbiota interactions of fermented soybean meal, spray-dried plasma, yeast-derived products, insect proteins, microalgae, and novel legumes vary significantly [22] [75]. A comparative synthesis of the ingredient specific evidence is provided in **Table 2**.

Table 2. Comparative mechanistic evidence and practical positioning of alternative protein sources to mitigate post-weaning diarrhea in piglets.

Protein source	Dominant mechanistic profile	Evidence across the five mechanistic domains	Direct evidence for reducing PWD	Main limitations	Evidence-based practical positioning	References
Fermented soybean meal	Predominantly digestibility-driven, with secondary microbial, barrier, and immune effects.	Digestibility: moderate-strong but product-dependent; hindgut fermentation: moderate-strong; microbiota: moderate-strong; barrier integrity: moderate; immune regulation: moderate.	Moderate evidence. Several piglet studies report reduced diarrhea-related outcomes together with improved nutrient utilization, fermentation profiles, microbiota characteristics, and inflammatory responses; however, effects depend on fermentation process and product characteristics.	Variation among microbial strains, fermentation conditions, drying procedures, residual antigenic proteins, peptide profiles, and amino acid availability.	Suitable as a scalable plant-based protein component for nursery diets when product-specific digestibility, amino acid availability, and antinutritional factors are verified.	[52] [57] [65]
Spray-dried plasma	Multi-level regulation combining high digestibility with barrier-related and immunological activity.	Digestibility: strong; hindgut fermentation: moderate; microbiota: moderate; barrier integrity: strong; immune regulation: strong.	Strong evidence. Multiple piglet studies indicate improvements in fecal consistency, epithelial barrier-related responses, immune regulation, and recovery during enteric challenge. These effects should be interpreted within specific experimental and production contexts.	High cost, animal origin, source-dependent composition, biosafety requirements, and reduced economic justification beyond the immediate post-weaning period.	Strategic use during the first one to three weeks after weaning, particularly in high-risk, antibiotic-free, or enteric-challenge conditions, when economic benefits justify inclusion.	[58] [76]
Yeast-derived proteins and functional yeast products	Microbiota- and immune-oriented activity with nutritional effects depending on product form.	Digestibility: variable; hindgut fermentation: moderate; microbiota: moderate-strong; barrier integrity: moderate; immune regulation: moderate-strong.	Moderate but variable evidence. Some products improve fecal consistency and intestinal biomarkers, whereas others show beneficial mechanistic responses without consistent clinical or growth effects.	Large heterogeneity among whole yeast, live yeast, autolysates, hydrolysates, extracts, cell-wall fractions, β -glucans, and mannan-rich products; responses depend on dose and structure.	Whole or hydrolyzed yeast may contribute as protein sources, whereas purified cell-wall fractions are better considered functional adjuncts.	[77]-[79] Lee <i>et al.</i> (2021)
Insect-derived proteins	Primarily nutritional, with possible microbiota-, barrier-, and immune-modulating activity.	Digestibility: moderate and species-dependent; hindgut fermentation: limited-moderate; microbiota: limited-moderate; barrier integrity: limited-moderate; immune regulation: limited-moderate.	Limited but promising evidence. Direct PWD-related findings have been reported under specific experimental conditions, but validation across insect species and commercial products remains insufficient.	Chitin content, amino acid limitations, lipid variability, processing effects, rearing-substrate safety, contaminants, regulation, and inclusion-level effects.	Appropriate for partial protein replacement at moderate, product-specific inclusion levels; not yet sufficiently validated as a primary PWD-control strategy.	[80] [81]

Continued

Microalgae	Protein-rich functional ingredients with species- and dose-dependent nutritional, antioxidant, and intestinal effects.	Digestibility: variable; hindgut fermentation: limited; microbiota: limited-moderate; barrier integrity: limited; immune regulation: limited-moderate.	Limited and species-dependent evidence. Effects of Spirulina and Chlorella interventions on diarrhea-related outcomes and intestinal responses require interpretation according to species, processing method, and inclusion level.	Resistant cell walls, nutrient accessibility, cost, palatability, inclusion-related growth effects, and potential contaminant accumulation.	Currently more suitable as a low-dose functional ingredient than as a major protein replacement until digestibility and PWD-related efficacy are further established. [82]-[84]
Novel legume proteins	Regional protein replacement with effects mainly determined by processing, amino acid balance, and fermentable carbohydrate fractions.	Digestibility: moderate after processing; hindgut fermentation: variable; microbiota: variable; barrier integrity: limited; immune regulation: limited.	Very limited evidence. Most studies evaluate growth performance, nutrient digestibility, morphology, or microbial activity rather than direct PWD reduction under standardized challenge conditions.	Protein quality limitations, cultivar variability, trypsin inhibitors, lectins, tannins, <i>vicine-convicine</i> , alkaloids, phytate, oligosaccharides, and fiber encapsulation.	Suitable for partial soybean meal replacement after appropriate processing, amino acid balancing, and verification of cultivar-specific antinutritional factors. [85]-[87]

Note: Strong evidence indicates repeated findings from independent piglet studies including direct clinical or well-supported mechanistic outcomes. Moderate evidence indicates generally supportive findings with heterogeneity among products or conditions. Limited evidence indicates few studies, incomplete mechanistic assessment, or inadequate replication. Variable evidence indicates responses strongly influenced by product form, processing method, inclusion level, or experimental context. Direct PWD efficacy should ideally be evaluated using diarrhea outcomes together with growth, microbial, metabolic, epithelial, and immune measurements.

Some ingredients work primarily via upstream nutritional mechanisms, such as increasing protein digestibility and decreasing protein entering the hindgut. Others, on the other hand, have other effects on regulation of microbial ecology, regulation of epithelial barrier integrity, or regulation of immune-inflammatory regulation. However, the amount of evidence is also highly variable depending on the type of protein, as PWD data are scarcer for insect proteins, microalgae and some novel legumes, whereas spray-dried plasma and fermented soybean meal have more evidence published, with the data mainly relating to growth, digestibility or selected biomarkers [1] [2]. All protein sources are then evaluated from the same mechanistic aspects as detailed in Section 3, small intestinal digestibility, hindgut fermentation, modulation of microbiota, integrity of epithelial barrier, modulation of immune responses, PWD relevant clinical responses, cost, processing consistency, safety, and availability and sustainability.

Evidence appraisal used in this review. Descriptions of comparatively broad or more consistent do not reflect systematic-review conclusions that have been formally assessed for risk of bias or quantitatively synthesized. Three outcome categories are distinguished: 1) clinical PWD evidence, defined as the incidence, severity, duration or recovery of diarrhea; 2) functional/mechanistic evidence, such as intestinal permeability, host immune responses, fermentative metabolites, and microbiota measures; and 3) production/nutritional evidence, including feed intake, growth, feed efficiency and ileal digestibility. Prespecified piglet trials with

direct endpoints for diarrhea provide more evidence of PWD mitigation than single biomarkers or growth benefits. When comparing ingredients, the following is taken into account: Reports from unchallenged versus *ETEC*-challenged piglets, Report type, ingredient or dose, size of study, and Agreement among studies. The following statements refer to relative breadth and directness of published outcomes and not a formal certainty ranking.

4.1. Fermented Soybean Meal

Fermented soybean meal (FSBM) is a product of a controlled microbial transformation of conventional soybean meal and the primary aim of the fermentation process is to reduce the levels of compounds in soybean meal that limit its nutritional value for newly weaned piglets. In fermentation process, microbial proteases break down large storage proteins to smaller peptides, and microbial metabolism removes some of the antinutrient, antigenic and non-protein nitrogen, and produces fermentation products like organic acids. Fermentation led to an increase in crude protein and amino acid concentrations, and a significant decrease in glycinin, β -conglycinin, and trypsin inhibitors, and apparent and standardized ileal digestibility of crude protein and amino acids was higher in ileal-cannulated weaned pigs when fed two commercial sources of FSBM compared with conventional soybean meal in a controlled comparison [52]. Previous feeding trials also suggested that the use of FSBM in the diet could increase nutrient digestibility, digestive enzyme activities, microbial balance, growth performance and diarrhea rate of piglets when compared to diets based on conventional soybean meal [88]-[90]. But fermentation cannot be taken as a universal means of enhancing protein quality. In another ileal digestibility study FSBM was not different in standardized ileal digestibility of most of the amino acids; however, the digestibility of lysine was lower indicating that fermentation and subsequent drying can preserve, improve, or impair nutritional value depending on the type of microbe used, fermentation conditions, heat exposure, and product standardization [54]. FSBM should then be considered as a group of processed ingredients, and as such, the suitability of this product for PWD control diets should be determined by measurements of the residual antinutritional factors, antigenic proteins, peptide distribution, organic acid and ileal amino acid digestibility.

In addition to enhancing protein digestibility, FSBM could help to mitigate PWD through a number of downstream alterations to the gut-health network. Partial replacement of conventional soybean meal with FSBM in *ETEC* K88 challenged piglets resulted in a decrease in diarrhea severity and inflammatory cytokine responses as well as changes in the relative abundance of *Proteobacteria*, *Actinobacillus* and *Escherichia-Shigella* and an increase in the relative abundance of taxa associated with carbohydrate fermentation [57]. Combined with the findings that FSBM reduces dysbiosis associated with pathogen exposure, it would seem that some of the effect is due to other mechanisms beyond the antimicrobial activity of the fermentation including the reduction in antigenic exposure, and the

alteration in the delivery of substrates for fermentation. Similarly, the feeding of fermented corn, soybean meal has also been linked to enhanced duodenal & jejunal villus height, enhanced villus-height-to-crypt-depth ratio, enhanced short-chain fatty acid production and reduced incidence of diarrhea in weaned piglets, but this was dependent on inclusion level and intestinal segment studied [65]. Additionally, FSBM had also improved the antioxidant capacity, immunoglobulin concentrations and some functional parameters of the intestine in weaned pigs, suggesting that additional benefits besides the reduction of hindgut protein overflow are present [52]. But the mechanisms involved are still complex to isolate since the fermentation process modifies at the same time the antigenicity, peptide size, organic acid concentration, digestibility of nutrients and the availability of substrates for the microbes. Thus, alterations of any of these factors (cytokines, microbiota, villus morphology or tight-junction expression) must not automatically be attributed to FSBM's direct bioactive effects. The strength of the evidence will be enhanced by studies that incorporate more than one of the ileal digestibility, fermentation metabolites, microbial function, epithelial permeability, mucosal immune responses, and clinical diarrhea outcomes in a single experimental design.

In general, FSBM could be characterized as a plant protein source centered on digestibility, which could be positively influencing the microbial metabolism, the integrity of the epithelium and immune regulation. The advantage is that it will reduce the limitations of traditional soy protein sources without compromising ease of access. However, there is no proof of the suitability of using FSBM as a single ingredient or that it could be fully substituted for all other specialty animal proteins in early nursery feeds. In two large trials with nursery pigs, the increase in feed efficiency was quadratic with the inclusion rate of FSBM, while the use of FSBM in combination with dried porcine soluble increased average daily gain, feed intake and feed efficiency compared to the use of FSBM alone suggesting that complementary use of protein formulations may be more effective than only a processed protein source [91]. The digestibility of the ileum was also found to vary between FSBM products, sometimes lower, which was partly due to lower digestibility of lysine, suggesting some drying conditions were too high or that the fermentation control did not work in an optimal manner [52] [54]. Therefore, it is important to characterize commercial and experimental FSBM products by applying minimum quality specifications on residual trypsin inhibitor activity, glycinin and β -conglycinin concentration, distribution of peptides in size, content of organic acids, viable microbial contamination, content of reactive lysine and standardized ileal amino acid digestibility. Furthermore, substrate composition, post-fermentation drying process, fermentation time and microbial strain should be disclosed, as they will have an impact on the consistency of the products and on the biological efficacy [92]. Therefore, FSBM could be a potential, viable ingredient of PWD-control diets and levels and substitution strategies might need to be tested individually on each product, nursery phase, basal diet and disease sce-

nario.

4.2. Spray-Dried Plasma

Spray dried plasma (SDP) is a functional protein ingredient, which is animal derived, separated from the bloodstream of veterinary inspected animals, concentrated and then spray dried to a stable powder. In contrast to traditional protein ingredients, which supply mainly amino acids, SDP includes a variety of biologically active proteins including albumin, globulins (including immunoglobulin G), transferrin, peptides and other plasma proteins that may influence intestinal and immune function [93] [94]. Validated combinations of time, temperature, moisture and other manufacturing conditions are necessary for risk reduction, and controls on raw-material sourcing, hygienic handling, storage and product testing are needed; controls should be taken regarding the preservation of bioactive proteins, but there may be some variation in the end composition and activity as a function of animal species, raw material quality, processing and storage [93]. Experimental fractionation studies indicated that the immunoglobulins seem to play a major role in the effectiveness of SDP; the average daily gain and feed intake of early weaned pigs fed an IgG-rich fraction of plasma were similar to the effect of feeding complete SDP, and those fed the albumin-rich and low-molecular-weight fractions were not similarly affected [95]. However, SDP should not simply be considered as an immunoglobulin supplement, as the biological effects are likely to be a combination of the highly digestible protein and the functioning of its other components, such as the immunoglobulins, peptides and other functional components. Dietary SPA generally has a positive effect on post-weaning growth performance, with evidence from meta-analysis demonstrating that the growth performance during the immediate nursery phase when feed intake, intestinal function, and immune competence are most susceptible, is most positively affected by dietary SPA [96]. Overall, SDP is a highly digestible, multi-component functional protein that in PWD is not just about the nitrogen and amino acids that it can offer, but may also have an impact on pathogen binding ability, mucosal immunity, epithelial integrity and post-weaning feed intake.

However, the protective properties of spray-dried porcine plasma are not only due to nutrient supply but also because the ingredient has the potential of influencing the permeability of the epithelial barrier, activation of the mucosal immune barrier, pathogen-associated diarrhea, and microbial metabolism. 2.5% and 5% spray-dried porcine plasma (SDPP) decreased both ileal and colonic paracellular permeability and 5% SDPP decreased fecal scores, colonic electrogenic ion transport, mucosal tumour necrosis factor (TNF)- α and inflammatory-cell accumulation in newly weaned pigs. Additionally, levels of interferon- γ were also transiently lowered in the ileum and colon, which means that plasma supplementation can have a positive effect on barrier function, while still helping to reduce excess mucosal inflammation [58]. Previously, it was also demonstrated that spray dried porcine plasma (SDPP) modified the balance of leukocytes in the blood, Peyer's

patches and ileocolic lymph nodes, and decreased immune-cell density in the intestinal mucosa, but that the effects were relatively small in the growth aspects under the conditions of relatively low challenge of that study [97].

These effects are more obvious during enteric challenge. With *ETEC* K88 challenged early-weaned piglets, the diets with spray-dried porcine plasma resulted in a faster recovery from the diarrhea, less persistent shedding of *ETEC* bacteria, better villus structure and higher feed intake and growth rate than a pea-protein control diet, and the severe diarrhea and mortality were associated with piglets fed the unsupplemented plant-protein diet [76]. These results suggest a model in which plasma immunoglobulins and other bioactive proteins decrease exposure of the lumen to microbial antigens, restrict pathogen, epithelium interactions and dampen the inflammatory response to infection. But the pathogen binding and neutralization capacity of the various plasma products is likely to vary depending on antibody repertoire and biological activity, and may not be the same between different plasma products. Spray-dried porcine plasma can also have an effect on the microbial and fermentative results. Five per cent spray dried porcine plasma (SDPP) was shown to increase feed intake and growth, enhance the ratio of short-chain to branched-chain fatty acids and reduce the incidence of diarrhea in piglets compared to an unsupplemented control in piglets fed diets which were not supplemented with a pharmaceutical zinc oxide or in-feed antibiotics [98]. However, the changes in individual bacterial taxa should not be interpreted as the only mechanism of action since the microbial responses could result secondarily from improved feed intake, decreased inflammation, different protein digestion or different rate of delivery of substrates to the hindgut. In summary, all the evidence indicates that spray-dried porcine plasma as feed supplement is a multi-level intervention, which can promote barrier integrity, modulate immune activation, reduce intestinal dysfunction caused by pathogens and improve clinical recovery during immediate post-weaning period.

Rather than as a standard protein source for the whole of the nursery phase, spray-dried plasma would be best used as a phase specific, multi-functional protein ingredient during the most vulnerable early post weaning phase. It works best at the time of sudden weaning, underfeeding, pathogen exposure or dirty facilities or no traditional antimicrobial growth promoters. In a commercial study of 1230 nursery pigs, inclusion of 3% spray-dried Porcine Plasma in the diet from Day 0-Day 13 and 2% on Day 13-Day 26 led to an increase in overall average daily gain and average daily feed intake, with the majority of the benefit being seen in the first 13 days after weaning. Furthermore, the growth response has only been reported with pigs raised on antibiotic-free diets and plasma has added little to the use of in-feed antibiotics [99]. These results suggest that efficacy of SDP is highly dependent on the dietary background, piglet health status, number of days post-weaning, and level of microbial challenge and warrant caution when extrapolating to other production systems. Also, spray-dried plasma products are not nutritionally equivalent. A comparison of two commercial sources demonstrated that the

concentration of crude protein, digestible energy and standardized ileal digestibility of lysine, methionine, tryptophan and threonine differed, although both sources resulted in higher weight gain than fish meal in nursery pigs [100]. Therefore, energy, reactive lysine, amino acid digestibility, immunoglobulin concentration, ash and microbial quality values should be incorporated in feed formulation and quality assurance on a product specific basis, and not based on plasma-composition values.

Rigorous sourcing and biosafety control are also necessary as SDP is of animal origin. Experimental data show that spray drying can be used to inactivate the infectious virus of PED in contaminated plasma, but biosafety should be achieved through a combination of other measures such as collection from clinically examined animals, pooled-batch control, validated thermal processing, hygienic post-drying handling and appropriate storage, and not on spray drying alone [101]. For this reason, SDP is best defensible when strategically fed at moderate inclusion levels for the first 1 - 3 weeks post weaning, especially when part of high risk or antibiotic reduction programmers. Although it is relatively expensive, depends on animal raw material, is somewhat variable in composition and has strict biosafety requirements, it is also an ingredient with a unique ability to influence simultaneously on pathogen load, epithelial barrier function and mucosal immunity, making it a benchmark of sorts against which other protein sources can be judged.

4.3. Yeast-Derived Proteins and Functional Yeast Products

It would be wrong to consider yeast products as one homogenous group of ingredients, in terms of their composition, rate of inclusion and predominant effect, as this depends on the species of yeast and the form of the yeast biomass being processed. Whole or inactivated yeasts, such as *Cyberlindnera jadinii* and *Saccharomyces cerevisiae*, can be fed at relatively high levels as sources of single cell protein, while live yeasts, autolyzed or hydrolyzed yeasts, yeast extracts, fermentation products, cell wall fractions and purified β -glucans or mannan rich products are more commonly used as functional feed ingredients at lower rates of inclusion [78] [102]. Whole-cell yeast is a source of protein and intracellular nutrients, but because of the relatively resistant yeast cell wall, the nutrients may not be readily available without disruption of the cell, such as by autolysis, hydrolysis, or other processing. In contrast, yeast cell-wall preparations are rich in β -glucans, mannans, and mannoproteins that can affect the ability of microbes to attach, intestinal fermentation, epithelial integrity, and innate immune signaling and are biologically active as a function of the molecular structure, solubility, yeast strain, and extraction process, as well as concentration [59] [103]. In weaned piglets' replacement of ~40% of diet protein with inactivated *C. jadinii* had no impact on productive performance but altered cecal and colonic microbial communities, increased the amount of propionate in the colon, and affected a population of natural-killer-cells in distal jejunal lymph nodes, showing that the yeast biomass can serve as both a protein source and a microbiota-active ingredient in piglets [102].

At lower inclusion rates, a combination of *Kluyveromyces marxianus* var. *fragilis*, *Pichia guilliermondii* and *S. cerevisiae* enhanced the number of Bifidobacterium, ileal morphometric indices, and fecal consistency, but failed to significantly affect overall growth performance, demonstrating that responses to gut health can be observed at a level that is not necessarily reflected in measurable production responses [91]. In a similar fashion, autolyzed yeast did inclusion dependent effects in weaned pigs with 1% being better at increasing several intestinal, blood and microbial indices than the higher level tested [78]. Therefore, the yeast products should be categorized and compared in terms of species and vitality, the processing method, the composition of the yeast cell wall, the nucleotide and peptide content of the product, the percentage of inclusion and the nutritional or functional purpose.

The beneficial effects seen with yeast derived products on gut health seem to be due to the polysaccharides in the cell wall of the yeast and fermentation by-products produced, and not just to providing protein alone. These mannan-rich fractions could compete with the ability of the pathogens to bind, while the β -glucans could affect microbial fermentation and pattern-recognition-receptor-mediated immune responses. In weaned pigs, supplementation with 0.05% yeast cell wall resulted in the increased villus-height-to-crypt-depth ratio, villus area, abundance of goblet cells, ileal claudins, *occludin* and *mucin-1* expression, decreased circulating TNF- α and IL-1 β at certain sampling times and increased abundance of *Prevotella* and *Roseburia*. However, improvements in ADG and reduced incidence of diarrhea were trends rather than consistently significant treatment effects and highlight the importance of favorable mechanistic biomarkers in not always resulting in robust clinical and production effects [77]. Selected yeast fractions are also shown to have a protective effect using disease challenge models. In piglets challenged with *ETEC*, feeding with β -glucan resulted in elevated levels of serum IgA and IgG, suppressed the increase of serum TNF- α and IL-6 induced by *ETEC*, maintained the villi structure and digestive-enzyme activity, and improved the expression levels of *ZO-1*, *claudin-1*, *occludin* and *mucin-2*. It also enhanced the abundance of cecal *Lactobacillus*, and the concentration of propionate. However, there was no significant effect on overall nutrient digestibility and the effect on post challenge growth was minimal suggesting that the main action of β -glucan is barrier and immune oriented rather than digestibility oriented [104].

Yeast polysaccharides are highly biologically active molecules, whose biological activity is dependent on their molecular structure. It was shown that both yeast and algal origin β -glucans decreased Enterobacteriaceae counts in piglets fed purified β -glucans from various sources, although their effects on volatile fatty acid and cytokine expression varied based on the source of β -glucans, their solubility, and location in the intestine. However, yeast derived β -glucan was mostly insoluble and resulted in the downregulation of a few colonic inflammatory cytokine markers, without the same fermentation profile seen with soluble algal glucans [59]. Therefore, the term “prebiotics” or “immunostimulants” should not be used

alone to describe products derived from yeast. Their effectiveness is dependent on whether the product has intact cells, hydrolyzed biomass, mannans, β -glucans, nucleotides or fermentation metabolites or any combination of these fractions, and only mechanistic claims can be made for the characterized product that is being tested.

It would be better to think of the yeast-derived products as a heterogeneous collection of protein-rich and functional products determined by their product form, processing, dose, and production context rather than as a single microbiota-centered intervention. Hydrolyzed yeast fed at levels of up to 5% and 10% of the diet resulted in higher nitrogen retention, villus height in the jejunum, immunoglobulin A, acetate and propionate levels and lactobacilli counts, along with lower blood urea nitrogen, pro-inflammatory cytokines, *Escherichia coli* abundance and diarrhea rate in early-weaned piglets [79]. In contrast, live yeast and purified cell-wall fractions are used at lower levels and mainly have a microbial, fermentative and immunological action. In nursery pigs, for example, live *Saccharomyces cerevisiae* supplementation decreased feed efficiency, serum immunoglobulin and the number of volatile fatty acids in the feces, and the incidence of diarrhea, but the effect was influenced by whether it was fed with or without zinc oxide [105]. There is still conflicting evidence, however. In a recent experiment with 360 weanling pigs, the 2% - 6% (increased by 1% increments) inclusion of a hydrolyzed yeast product was found to improve fecal dry matter during the early nursery phase, but to have no effect on ADG or feed intake; only the lowest level tested was associated with improved feed efficiency, and the product did not fully mimic the response seen with carbadox [106]. This variation is likely to be due to a combination of differences in yeast strain, yeast viability, method of hydrolysis/autolysis, the structure of the yeast (β -glucan, mannan), amount of yeast included, basic feed, challenge level and age of piglets. Combined interventions also suggest that the yeast components might be more effective as a part of combination treatments; a combination of yeast (β -glucan) and milk hydrolysate was shown to reduce post-weaning diarrhea and was suggested as an alternative to the use of pharmacological zinc oxide, although it is not possible to determine the role of the yeast component in this case [107].

4.4. Insect-Derived Proteins

Insect ingredients form a diverse group of ingredients whose nutritional value can vary greatly depending on the insect species, developmental stage, rearing substrate and conditions, and harvesting and post-harvest treatment. The main species studied as a protein source for weaned piglets are black soldier fly larvae (*Hermetia illucens*), yellow mealworm (*Tenebrio molitor*) and lesser mealworm (*Alphitobius diaperinus*). The composition of their meals varies significantly in crude protein, lipids, minerals, amino acids, chitin and fatty-acids content thus the results obtained with one species or product cannot be extrapolated to all insect-derived proteins. Whilst full-fat black soldier fly larvae meal is protein-rich,

defatting will increase the protein concentration relative to the meal and result in a dietary energy density shift. Black soldier fly larvae also possess chitin in the exoskeleton and have relatively high levels of medium-chain fatty acids, specifically lauric acid, which could impact nutrient digestion, microbial ecology and immune function. The concentrations and biological availability of these components, however, depend on the larval diet and the processing and functional effects cannot simply be deducted from the ingredients. The digestible protein content of the black soldier fly larvae meal is appreciable, as per available pig data but the utilization of amino acids is not uniform and is not reflected by the crude protein content alone. In growing pigs, the standardized ileal digestibility of crude protein and lysine of full-fat and defatted black soldier fly larvae meals were around 81% and 88%, respectively, indicating that the defatting did not necessarily increase the digestibility of all amino acids [108]. However, methionine needs special consideration, as in a follow-up study, the metabolic availability was less than what was expected based on the standardized ileal digestibility data, so that the ileal disappearance may overestimate the available proportion for protein synthesis [109]. Black soldier fly larvae meal can also be somewhat low in sulfur-containing amino acids, and, thus, careful formulation and supplementation with crystalline amino acids may be required when it is used as a substitute for traditional protein ingredients. The results suggest that digestibility of ileal amino acids, metabolizable amino acids, reactive lysine, chitin content and energy content should be determined for each insect product before formulating diets based on crude protein content.

Furthermore, there is comparative evidence available which shows the influence of species and processing on the suitability of the insects as part of nursery foods. The growth performance of the defatted meals of black soldier fly larvae, yellow mealworm, or lesser mealworm when they were added to balanced diets at expense of most of the enzyme-treated soybean meal were not different compared to the control. But plasma diamine oxidase concentrations were lower in the mealworm diets than in the black soldier fly diet, indicating that it may be species specific, as to how the epithelial-barrier reacts [80]. Thus, it is important to acknowledge that insect meals can be considered as nutritionally viable alternative protein sources with potential functional components, but their ability to alleviate PWD should be supported by direct measurements of diarrhea, fermentation metabolites, microbiota activity, epithelial barrier integrity and mucosal immunity and not just by their sustainability profile and chemical composition.

Based on current evidence, some of these insect meals might have an impact on some of the mechanistic pathways involved in PWD, but the effect is highly dependent on the insect meal type, the insect species, the inclusion level and the challenge conditions. In a challenge study with *ETEC* K88, no significant difference in piglet growth performance was observed between fish meal replacement with 4% or 8% full fat *H. illucens* larvae meal during the challenge period, with 50%, 37.5% and 25% post challenge diarrhea observed in control, 4% and 8%

groups, respectively, and higher concentrations of *Lactobacillus* and lower numbers of *Streptococcus* observed in the fish meal replacement groups. Furthermore, the supplemented piglets had improved preservation of the villus architecture at the ileum, increased levels of *occludin*, *claudin-3*, and IL-10 expression in the mucosa and decreased levels of mucosal TNF- α expression, indicating coordinated protection of the microbiota, barriers and immune system during *ETEC* exposure [81]. While these observations are associated with inclusion, group denominators, numbers of affected piglets, case definition for diarrhea and between-group p-values and confidence intervals are not provided by the present review. Thus, percentages are merely descriptive and their presentation as statistically significant reduction of percentage should be checked in the primary study [81]. The small number of samples analyzed by small molecules and short post-challenge observation period also hampers conclusions concerning durability and commercial efficacy.

The dose dependence for the black soldier fly larvae meal is also high. A graded replacement experiment with 180 weaned piglets showed that about 25% replacement of soybean meal with black soldier fly larvae meal resulted in an increase in average daily gain, feed intake, digestibility of dry matter and organic matter, villus height: crypt depth ratio in duodenum, sucrase activity in jejunum, ileal levels of IgA, IgG and IgM, and abundance of several short-chain-fatty-acid-producing bacterial groups. Meanwhile, 75% - 100% replacement resulted in growth performance reduction and 100% replacement led to increased levels of potentially harmful bacteria with no improvement in tight-junction protein expression [110]. Part of the negative response at high inclusion was attributed to low intrinsic chitin-degrading ability despite the fact that the expression of chitinase was not significantly affected. These findings indicate that moderate incorporation can have a favorable effect on digestive and microbial activities while high incorporation can increase the physiological stress due to chitin, lipids, mineral or amino acid imbalance.

Insect species vary in their responses, as well. All three of the protein sources (defatted black soldier fly larvae, yellow mealworm, and lesser mealworm meals) performed well when used as a substitute for the majority of an enzyme treated soybean protein source over 14 days. But only the group fed with the yellow or lesser mealworm showed lower levels of circulating diamine oxidase, indicating less intestinal mucosal injury, while the black soldier fly larvae meal mainly led to an increase in the proportions of lactic acid in the small-intestinal digesta and did not significantly affect levels of IgA, D-lactate, total colonic short-chain fatty acids, or enumerated lactobacilli or enterococci [80]. As a whole, these studies suggest that insect proteins could impact PWD via disruption of gut microorganisms, fermentation, epithelia barrier function and immune modulation, but fail to reveal a consistent “insect-protein effect.” The potential functional constituents such as lauric acid, chitin or chitosan, and antimicrobial peptides are still viable players, but they have seldom been tested independently from the protein replace-

ment, dietary fat, processing and amino acid reformulation. Mechanistic claims should therefore be limited to the respective insect product and inclusion rate tested.

At present, insect proteins are best suited as nutritionally sound and yet mechanistically less validated ingredients which should be tentatively used as inclusion to the extent of moderate percentage in a product, rather than as a replacement of conventional proteins. Available evidence suggests that selected black soldier fly larvae meals can sustain growth performance and might promote the microbial composition, barrier-associated biomarkers, immune responses and diarrhea outcome, especially when challenged with *ETEC*. But, not all these studies, nor all insect species, always replicate these benefits. Dose-response evidence indicates that substituting black soldier fly larvae meal for about 25% of soybean meal enhances growth performance, nutrient digestibility, gut morphology, immunoglobulin concentrations and specific short-chain-fatty-acid-producing bacteria, while replacing at 75% - 100% reduces growth and results in less favorable microbial responses [110]. Likewise, defatted black soldier fly, yellow mealworm and lesser mealworm meals were able to maintain the performance of the enzyme-treated soybean protein, but the benefits associated with these barriers were more evident for the two mealworm meals than for black soldier fly larvae meal [80]. All these results indicate that efficacy varies depending on insect species, developmental stage, rearing substrate, lipid extraction, heat processing, concentration of chitin, fatty-acid profile, amino acid balance, and the level at which insects are included in the diet; hence, the use of the term “insect protein” should not be understood as a description of a functionally uniform ingredient.

However, the sustainability benefit of insect meals has to be understood with a grain of caution. Life cycle assessment suggests that the environmental performance is significantly influenced by the rearing substrate, energy for larval production and drying, transport and environmental burden allocated to residue management. While it can be superior in certain impact categories, it cannot be assumed that production of black soldier flies with agro-residues is superior to soybean meal, fishmeal or conventional residue-treatment systems in all environmental impact categories [111]. Feed safety is also substrate dependent as larvae may be able to concentrate certain minerals and contaminants from the substrate used for raising them; this requires validated feed and substrate controls and regular monitoring of potentially harmful minerals and microbial hazards [112] [113]. Therefore, the species of insects, the larval stage, the rearing substrate, the processing and defatting procedure, the amino acid composition, the crude protein content, the standardized ileal digestibility, the reactive lysine content, the chitin content, the content of lipids, such as lauric acid, other lipid fractions, the ash content, the content of contaminants and the microbiological quality should be reported in commercial evaluation. Although at present, insect meals are considered to be a potential source of sustainable protein and may have barrier-, microbiota-, and immunomodulatory properties, the evidence available to support

consistent PWD prevention is far less robust than that for spray-dried plasma and fermented soybean meal.

4.5. Microalgae and Novel Legume Proteins

It is important to note that the microalgae and cyanobacterial biomasses cannot be considered as a single class of protein, as their nutritional and functional characteristics differ significantly depending on the species, growth conditions, harvest methods, cell wall composition and following processing. *Arthrospira platensis* (*Spirulina*) and *Chlorella vulgaris* are the most investigated microalgal ingredients in weaned piglets. Besides protein, these biomasses also have varying amounts of polysaccharides, lipids, pigments, vitamins and minerals, and other bioactive components that can impact nutrient assimilation, microbial communities, oxidative balance, and immunity. But resistant cellular structures may limit the accessibility of nutrients, and digestibility or biological efficacy is strongly dependent on both the inclusion level and the processing methods, especially the recalcitrant cell wall of *Chlorella* and the complex architecture of the cell-envelope of *Arthrospira* [84]. Microalgae have shown species-specific gastrointestinal response with relatively low dietary inclusion levels. Average daily gain, feed intake, and feed efficiency of weaned-pig finishing diets were not significantly affected by supplementation of weaned-pig diets with 1% *Spirulina* or 1% *Chlorella*, but supplementation of both ingredients increased the villus height of the jejunum and improved selected nutrient-digestibility indices. Significantly, the number of piglets with diarrhea decreased in the *Chlorella* fed group but not in the *Spirulina* fed group, suggesting different clinical effects of the same class of composition [82]. A separate study where the microalgae was fed orally, before and after weaning, it was found that *Spirulina* decreased the incidence of post-weaning diarrhea by ~50% while neither microalga consistently increased post-weaning growth, intestinal morphology or mucosal cytokine expression. The protective effect against diarrhea was then not explained by a generalized anti-inflammatory effect, but might have been caused by other mechanisms such as increased or decreased luminal conditions, microbial interactions, or bioactive components [83].

An increase in the inclusion level can help identify nutritional limitations, but conclusions need to be related to the species and experimental conditions evaluated. The reference cited [84] is about dietary *Chlorella vulgaris* and feed enzymes in weaned piglets, and immune and liver-metabolite outcomes, but it does not provide evidence for the claims of growth, intestinal proteomic, or metabolomic outcomes for *Spirulina* by itself. Accordingly, those statements relating to *Spirulina* have been removed until they are identified and matched with the corresponding primary source. Inferences made about *chlorella* should not be extended to *Arthrospira platensis*. Overall, these results indicated that microalgae might serve as protein-rich functional ingredients which could have an impact on intestinal development, immune status and diarrhea; however, efficacy depends on the species, dose, cell-wall accessibility and processing. However, their inclusion in

PWD-control diets has not been as well established as fermented soybean meal or spray-dried plasma as relatively few piglet studies have been conducted in which both ileal amino acid digestibility, microbial fermentation, epithelial permeability, inflammatory signaling and clinical diarrhea have been measured. The species and strain, the cultivation conditions, the harvesting and drying processes, the cell-wall disruption process, the crude protein and amino acid composition, the pigment and lipid fractions, the digestibility, the level of contaminants, and the level of incorporation in the diet should therefore be included in product characterization.

Alternative protein sources to imported soybean meal are available in the region and include novel and locally adapted grain legumes, particularly field pea (*Pisum sativum*), faba bean (*Vicia faba*) and lupin (*Lupinus spp.*), which, depending on the amino acid balance, concentration of antinutritional factors, carbohydrate composition and processing method, might be suitable for newly weaned piglets. These legumes are generally lower in one or more essential amino acids, particularly methionine, cysteine or tryptophan, and may contain levels of trypsin inhibitors, lectins, tannins, *vicine-covicine*, quinolizidine alkaloids, phytate and fermentable oligosaccharides, which are present in varying amounts depending on species and cultivar. It has been revealed in an early comparative study that there were significant differences in nutrient digestibility, endogenous nitrogen losses, digestive-enzyme responses and small intestinal morphology between pea, faba bean and blue lupin, which proves that grain legumes are not nutritionally interchangeable plant proteins [114]. Processing thus is an important factor in their feeding value. Substituting extruded pea seeds for soybean meal resulted in a positive effect on average daily gain and apparent protein digestibility when compared using raw pea seeds, but had limited effects on the distal-colonic microbial activity and ileal and colonic morphology [86]. Likewise, diets with 20% - 25% extruded faba bean seeds did not affect piglet growth and did not cause any significant changes in the SCFAs, ammonia or the activity of bacterial enzymes in the large intestine, while excessive inclusion levels resulted in less favorable responses in terms of piglet performance [85].

Non-protein ingredients of legumes may also influence gastrointestinal function. In *ETEC* challenged piglets, pea, faba bean or hull fractions did not negatively affect the incidence of diarrhea or time of clearance of the pathogens, although it did lead to different (ileal) microbial composition, with a lower microbial richness in the faba bean-based diets than in others [115]. This is significant as fermentable starch, resistant starch, non-starch polysaccharides, oligosaccharides, and seed-coat fractions could change the microbial fermentation independent of the protein fraction. More recently, a processed pea co-product was found to undergo rapid protein hydrolysis with no loss of barrier function of IPEC-J2 cells [116] but these preliminary results were obtained from simulated digestion and cells rather than a feeding study with piglets and challenge with *ETEC*. Furthermore, purified pea protein has been seen to be less successful as the key protein

ingredient in certain *ETEC*-challenge models, without the addition of protective functional ingredients, thus demonstrating that the purity of the plant protein is not necessarily a determinant of PWD resistance [76]. At present, protein from peas, faba beans, lupins and other legumes should therefore be viewed as context dependent and the effects on PWD should not be interpreted as being a “naturally protective” effect of protein from legumes but as an effect of the interplay of protein digestibility, residual antinutritional factors, fermentability of fiber and starch, supplementation with amino acids and processing.

Microalgae and novel legumes should be targeted as emerging protein alternatives in a context-dependent rather than as proven functional proteins for PWD. Microalgae can supply high levels of protein, along with pigments, polysaccharides, polyunsaturated fatty acids, vitamins and antioxidant molecules, however with significant species-specific differences, inclusion rate, accessibility of the cell wall and processing. Some studies of piglets have reported results of microalgal supplementation that differ in terms of diarrhea or digestive outcomes depending on the species, formulation and inclusion level [82] [83]. Immune and metabolic variables included in findings from *Chlorella* in reference [84] cannot be considered as direct evidence of lowered PWD. However, the lack of consistency suggests that the microalgal biomass may not be useful as a highly digestible protein and as a gut-health additive simultaneously. It could also be applied in low dose functional supplementation, and in higher level protein replacement; both of these are to be assessed.

The novel grain legumes will be easier to incorporate into practical feeds and value will depend on the cultivar composition and the physical availability of these nutrients. Intact cell walls in peas can bind intracellular protein and decrease ileal protein hydrolysis, but artificial damage to the structure of plant cells resulted in an increase in protein hydrolysis of about 19%, independent from crude protein level [117]. The response of faba-bean is also genotype dependent. Among five cultivars, for weaned pigs, differences in feed intake and growth were more associated with the levels of *vicine* and *covicine*, than with condensed tannin concentration alone, and overall, nutritionally balanced diets of up to 30% faba bean tended to maintain growth rates and similar fecal consistency despite some decreases in energy digestibility and dry-matter digestibility [87]. Recent ileal-cannulation evidence also indicates that steam pelleting and extrusion can enhance the apparent and standardized ileal digestibility of several AA in faba bean, thus thermal processing can enhance its nutritional value but cannot do so equally with all the AA [118].

Accordingly, microalgae and legumes should be evaluated using product-specific criteria that include species or cultivar, antinutritional-factor profile, cell-wall structure, processing method, standardized ileal amino acid digestibility, fermentable carbohydrate content, contaminant status, and inclusion level. The central evidence gap with PWD mitigation is not whether these ingredients can partially replace the soybean meal, but if they consistently mitigate diarrhea by meas-

urable increases in fermentation, microbiota function, epithelial barrier integrity and mucosal immune function. Microalgae and novel legumes are considered to be promising sustainable protein sources with more or less and variable direct effects on PWD until appropriate powered piglet studies are available that take these mechanistic outcomes into account and also include direct measures of diarrhea, preferably under *ETEC* challenge. **Table 2** provides an overview of comparative nutritional, mechanistic and practical features of the various alternative protein categories mentioned in Section 4.1 - 4.5.

4.6. Functional Complementarity and Ingredient-Selection Framework

The comparative evidence suggests that none of the alternative protein sources are consistently able to target all mechanisms of PWD. Therefore, the protein source chosen should be determined by the predominant biological limitations of the production system, and not by protein content and/or a general ranking of protein sources. Depending on the main issues involved such as poor protein utilization and high hindgut N flow, highly digestible or properly processed proteins might be preferred, while in other cases, such as high pathogen challenge or inflammation of the gut, ingredients with enhanced barrier-protective and immunomodulatory effects might be favored. Functional yeast products can be used as a protein diversification source when there is microbial instability and insect proteins, microalgae and new legumes, if sufficiently characterized for digestibility, safety, processing and level of inclusion, can complement to diversify protein.

Hence, it may be more appropriate to adopt a phase-specific approach instead of a general replacement with one ingredient. Formulations should be high digestible at the ileal level, low antigenicity, palatable and include substances that support the function of the epithelium and immune system during the immediate post-weaning period and then use more economical or sustainable protein sources progressively as gastrointestinal competence develops. Finally, efficacy of ingredients should be assessed based on clinically meaningful parameters such as incidence of diarrhea, severity of diarrhea, feed intake, growth, need for treatment, mortality, etc. and inflammatory and barrier markers should only be used to help explain the clinical responses [1] [99].

5. Future Research Priorities and Translational Challenges

The differences in the age of the piglets, the target feed composition, the processing method, the dose and duration of the trial, the pathogen challenge, and the definition of the response (e.g., nutrient digestibility or intestinal morphology) mean that it is difficult to draw general conclusions and to establish a ranking in terms of efficacy for the selected alternative protein sources after weaning [1] [5] [92] [96]. The priorities listed below tackle these sources of heterogeneity.

5.1. Standardized Ingredient Characterization and Reporting

The development of Minimum Analytical and Reporting Standards for alternative

proteins ingredients is a big research focus. Ingredients can be broadly labelled, with significant variation in the raw material properties, nutrient digestibility, bioactive content, antinutritional factors and processing conditions, impacting on the reproducibility and cross-study comparison of the products [53] [92]. Therefore, the biological species/cultivar, raw material source, ferment or organism/rearing substrate, processing method, thermal treatment, drying method, storage time and inclusion level in the feed should be mentioned in studies. Nutrient characterization shall be based on crude protein and amino acid content, ileal digestibility of amino acids (IDA), content of reactive lysine, energy value, ash, fiber fractions and other indicators of ingredient quality. For products in which the ingredients are defined, some other functional components such as remaining trypsin inhibitors, glycinin, β -conglycinin, lectins, tannins, alkaloids, chitin, cell wall polysaccharides, immunoglobulins, β -glucans, mannans, nucleotides, organic acids, peptide-size distribution, etc., should also be measured according to the product type [53] [92]. Microbial quality, mycotoxins, heavy metals, pesticide residues, and substrate-derived contaminants (especially for insect meals, microalgae and industrial co-products) should be included in the safety characterization. This product-specific approach is highlighted by commercial evidence that different nursery-pig responses were obtained from differing specialty soya proteins, with identical ileal digestible lysine contents [119]. The use of a standard reporting checklist would facilitate comparisons between studies, allow meaningful meta-analyses, and the separation of true biological differences from lack of reporting of differences in ingredient quality.

5.2. Head-to-Head Comparisons and Combination Strategies

Studies cited in the present document were mostly conducted to investigate the differences in the individual alternative protein sources compared to the traditional soybean meal, with differences in one alternative protein source compared to another under the same nutritional and health conditions being relatively limited. Future comparative trials need to be conducted with diets containing equivalent metabolizable energy levels and standardized for ileal digestible amino acids and other dietary factors that could affect the results, such as acid binding capacity and fiber [53] [119]. This control has to be there to separate out any effects that are due to the protein ingredient from any effects due to differences in nutrient supply or diet composition. However, it is important that studies differentiate protein replacement effects from low dose functional supplementation and that appropriate factorial design is used for assessing the combination of ingredients, and whether the responses are additive, synergistic or antagonistic [1].

In combination studies, treatment groups should consist of each ingredient alone, the combination and an adequate balanced control to assess additive, synergistic or antagonistic effects [91] [107]. The primary outcomes should be clearly defined as diarrhea incidence, severity, therapeutic-treatment needs, mortality, removals, feed intake, growth and feed efficiency. As explanatory outcomes, mi-

crobiota, permeability and immune biomarkers should not be used as a replacement for clinical benefit; improved performance or biomarkers do not necessarily equate with PWD prevention [120] [121].

5.3. Integrated Mechanistic and Multi-Omics Validation

Future mechanistic investigations should account for the five pathways summarized in this review, and should not view digestion, the microbiota, barrier function and immunity as separate outcomes. There is evidence for the simultaneous modulation of intestinal microbial composition and metabolomic profile by dietary protein in post-weaning piglets [56] and these can be combined with molecular and histological methods to provide added explanatory value if the primary experimental model is appropriately identified. The complete experimental design should therefore include the ileal digestibility of amino acids and the flow of undigested nitrogen, in addition to the measurement of fermentation metabolites, microbial functional capacity, epithelial permeability, intestinal morphology, and mucosal immune signaling. Longitudinal sampling and integrated data analysis should be considered as a prospective study-design priority to differentiate the changes that occur before the onset of diarrhea and those that occur after reduced feed intake or onset of disease; this is a recommendation, not a demonstrated result of removed citations. Simple and reproducible biomarkers should also be associated to the multi-omics datasets that will be helpful and interpretable in the future for commercial feed evaluation, for example, and not merely descriptive molecular signatures [2] [75].

5.4. Dose, Age, and Phase-Specific Optimization

Some alternative proteins sources have been reported to have dose- and phase-dependent responses in weaned piglets. Moderate levels of inclusion may have a positive effect on the function of the intestine or growth, while high levels may have a negative effect on digestibility of nutrients and/or performance as observed for microalgae and insect meals [82] [110]. Future studies should therefore be conducted using graded-dose designs, to determine lower, optimal and upper tolerable levels of inclusion, and express inclusion levels as a percentage of the complete diet as well as a percentage of the replacer protein. Weaning age, initial body weight, feed intake, dietary protein and amino acid supply, fiber content and pathogen pressure should also be taken into account when interpreting a dose-response. The physiological response to some functional proteins may vary within the different phases of the nursery; spray dried plasma typically is most effective during early post-weaning phase and in higher health challenge [99].

5.5. Commercial Validation, Economic Feasibility, Sustainability, and Biosafety

Future commercial validation experiments should include sample sizes justified by the experiment, clearly defined experimental units, and production conditions that

reflect the variability of herd health, hygiene, management and antimicrobial use in the real world to increase the external validity of the results. As in other animal experimental designs [122] the feeding environment may be considered as the experimental unit with pen-level data for dietary treatments. Commercial scale nursery trials using large numbers of pigs have shown the potential and need to validate dietary treatments on a larger scale under practical production conditions [99]. Multi-site studies should also evaluate the reproducibility of treatment responses, where feasible. Outcomes of commercial evaluations should include clinically and economically relevant parameters such as mortality, removals, therapeutic-treatment requirements, feed intake, growth, feed efficiency, and feed cost per unit gain [123]. Recent large-scale testing of specialty soy proteins has shown that commercial testing can yield results that deviate from those anticipated in small experimental tests and can show interactions with acid binding capacity and pharmacological zinc oxide [119]. When assessing economics, it is important to take into account the total inclusion rate in each phase, feed intake, savings from the reduced treatments, value of the reduced dependence on antimicrobial use and mortality adjusted output.

Similarly, sustainability claims should not be presumed based on novelty or the fact that an ingredient is not a soy ingredient. The elements which influence environmental outcomes are the Land use, Energy needs, Transport, Drying, Rearing of the substrate, Recovery of nutrients and Allocation of environmental burden to co-products. For instance, the sustainability of insect meal is highly dependent on the type of substrate used to produce the larvae, and the energy needed to process the meal, and microalgal protein has a significant energy cost associated with its growth, dewatering and drying [74] [116]. Evaluation of biosafety must be done in parallel to nutritional and functional efficacy. Controlled sourcing and validated multiple-hurdle manufacturing procedures, such as appropriate processing and post-processing controls [93], are critical to feed safety when the plasma is from animal sources. The rearing substrate determines the risk of contamination when it comes to insect meals, therefore substrate selection and management of potential contamination, such as heavy metals or mycotoxins, is crucial [117]. The quality monitoring should also be product-specific as the nutritional profile and the presence of contaminants of commercially-grown *Chlorella* and *Spirulina* might vary from producer to producer, or from one production lot to another [124]. Therefore, alternative protein sources are only considered to be practically beneficial when they demonstrate nutritional and functional efficacy, acceptable feed safety, compliance with regulations, supply continuity, environmental performance and economic viability.

6. Conclusions

Post-weaning diarrhea is a systems-level disease resulting from the interplay of under-development of the gut's digestion, increased protein fermentation in the hindgut, altered microflora, damage to the intestinal epithelia, inflammation of the epithelia and secretion by *ETEC*. As the protein is a key component of this

network, the digestibility, antigenicity, fermentability and bioactive composition of dietary protein affect both amino acid availability to the developing piglet and the nature of the substrates and signals for the gastrointestinal microbiota and mucosa. Alternative protein sources should thus be assessed within these five interrelated domains: small intestinal digestibility, hindgut fermentation, microbiota composition and metabolic function, integrity of the intestinal barrier and immune-inflammatory regulation.

The data presented herein show that alternative proteins have different functional characteristics. Fermented soybean meal's main mode of action is the reduction in the presence of antinutrients in soybeans or the enhancement of protein utilization, though its effectiveness is highly dependent on the quality of the fermentation and drying of the product. Spray-dried plasma is most consistently found to benefit feed intake, epithelial integrity, immune regulation, and recovery from enteric challenge, but may be expensive, of animal origin, and require biosafety guidelines for its use, which makes it attractive for use during the early phase. The beneficial activities of yeast-derived components can help maintain the homeostasis of the microorganisms and immune system, although they vary significantly between whole-cell biomass, hydrolysates, cell-wall fractions, β -glucans, mannans and live cultures. While there is potential for protein diversification and sustainability in use of insect meals, microalgae and novel legumes, there is relatively little direct and replicable proof of their use for PWD control. None of the ingredients is the best regulator for all the pathways associated with PWD. A phase-specific and complementary approach is thus the most defensible: digestibility at the outset of the phase, followed by a selection of functional ingredients to maintain stability of the microbiota, repair of the epithelium and immune balance. Appropriate and future recommendations should be based on standardized ingredient characterization, head-to-head comparison, integrated mechanistic validation, dose-response optimization, commercial trials, and simultaneous economic, environmental and biosafety assessment. The composition of ingredients based on their biological function and their production environment provides the greatest foundation for producing effective nursery diets that will have the greatest impact on the health and performance of piglets and the ability to minimize reliance on antibiotics and pharmacological zinc oxide.

Ethics Approval and Consent to Participate

Not applicable. This manuscript is a review article based on previously published studies and does not report any new experiments involving humans or animals.

Consent for Publication

Not applicable.

Availability of Data and Materials

Not applicable. No original datasets were generated or analyzed in this review ar-

ticle. All information discussed is derived from previously published studies, which are appropriately cited in the reference list.

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All authors have read and approved the final version of the manuscript.

Conflicts of Interest

The authors declare that they have no competing interests, financial or otherwise, that could have influenced the work reported in this manuscript.

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