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Clinical studies on the effects of functional feeds for cultured salmonids: a critical assessment of health indicators, results, experimental designs and statistical methods

A report sponsored by

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Index

Preface.....	3
Summary.....	4
Introduction.....	6
Material and working methods	7
Terminology and definitions.....	8
Overview of the published literature.....	11
Review papers.....	11
Cell wall polysaccharides (beta-glucans, mannan oligosaccharides)	12
Other immunostimulatory ingredients	15
Lipids, bile salts, organic acid salts, and others	17
Nucleotides	18
Prebiotics.....	22
Probiotics	23
Vitamins and carotenoids.....	27
Alleviation of feed induced intestinal pathologies.....	29
Summary assessment	30
Critical evaluation of research design and methods used in health feed trials	33
Are observational cohort studies the answer?.....	37
Suggestions for design and statistical methods to be used	38
Types of studies and their use.....	38
Design of controlled trials.....	40
Design of field trials.....	42
Statistical techniques.....	43
Meta-analysis	43
Further comments on design.....	44
List of attachments	45

Preface

This report was produced at the Centre for Epidemiology and Biostatistics of the Norwegian School of Veterinary Science (NVH) through a project financed by The Norwegian Seafood Research Fund (FHF). The project was initiated in order to scrutinise the claimed or perceived health effects of using specific diets to support disease control in salmonid fish farming. Due to current challenges in the control of several viral diseases (Pancreas Disease, Cardiomyopathy Syndrome, Heart and Skeletal Muscle Inflammation) and in the management of salmon lice, the need for new and/or supportive measures in fish health management is obvious. The report deals with the questions raised by first taking a deep dive into available literature, followed by an assessment of study design and statistical analyses employed in the published studies. The report concludes with suggestions on design and statistical analysis focusing on the assessment of clinical health effects in the real field situation.

The project has been carried out by researcher Paul J. Midtlyng (NVH), in close collaboration and co-ordination with professor Eystein Skjerve (NVH). Substantial input was given by associate professor Anne Marie Bakke (NVH) and Ph.D. student Leon Cantas (NVH). During the work, statistical expertise was provided by Professor Stig Larsen (NVH) and visiting Professor Tim Carpenter (University of California, Davis). The steering group was consulted and asked for input throughout the process. We thank all of you for the good cooperation. The final report and conclusions are written by Paul J. Midtlyng and Eystein Skjerve and critically evaluated by Anne Marie Bakke, who will jointly pursue the publication of the work in a renowned international journal. We hope the report will be read, used, and criticized.

Oslo, May 2012

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Summary

This report, sponsored by the Norwegian Seafood Research Fund (FHF), aims to summarise and scrutinise the data underlying the current use of specific diets (functional feeds) to support fish health management and disease control in salmonid aquaculture. In addition to a scientific overview of published scientific articles, the report discusses experimental designs and statistical and biomedical methods used in these studies, and provides recommendations on study setups and statistical methods to document health effects of fish feeds.

There is a striking abundance of review papers and overview publications covering the health effects of diets, in particular dealing with probiotics (24 papers), followed by various immunostimulants including beta-glucans (21 papers). Among 152 published papers providing original results from studies of *in vivo* effects derived from nutritional interventions, the majority report indirect outcome measures of health, predominantly immune parameters such non-specific immune responses, immune cell activity, antibody levels, or expression of various immune genes. Beneficial effects of cell wall polysaccharides (including beta-glucans), nucleotides and vitamins/carotenoids have been shown in a limited number of controlled disease challenge trials with Atlantic salmon and rainbow trout. However, support for these observations from field trials is apparently absent with the exception of one study involving beta-glucan administration to Atlantic salmon.

Various categories of probiotics (lactic acid bacteria, aeromonads/ pseudomonads, micrococci/enterococci) have shown beneficial clinical effects in rainbow trout challenge trials, while support for moderate beneficial effects of probiotic yeasts are mostly derived from field studies. Despite the widespread use of proprietary functional feed ingredients or formulas, there are only a dozen papers addressing their clinical effect among the reviewed literature.

Administration of feed to farmed salmonids must take place in groups, and cannot be measured or even estimated individually without exceptional efforts. As a consequence, the group in a pen or tank collapses into one single unit of concern for statistical purposes. However, a main assumption behind standard statistical techniques is the independence between study subjects. Many of the refereed studies nevertheless rely upon parallel group design with simple parametric or non-parametric group comparisons or ANOVA-based methods using measurements conducted on individual fish.

The fact that infectious diseases spread between individuals in a group, and hence the criterion of independence between individuals is typically broken, applies to controlled studies as well as to field trials. In experimental or natural disease situations alike, individual animals will always infect each other and specific statistical techniques are needed in order to address this situation adequately.

Based upon the discussion we bring up in this report, we suggest that documentation of clinical effects from functional fish feeds should comprise:

1. Solid support for relevant disease-specific benefits in experimental studies.
2. Clear prevention or modification effects shown in a controlled disease challenge trial setup.
3. Final documentation and quantification of clinical effects from large-scale field trials/cohort studies.

Examples of both simple and more complex designs of controlled or field trials are provided and it is strongly recommended to employ various multivariable regression techniques in order to adjust for group effects or other design issues when analysing data from such studies.

Introduction

Aquaculture is the fastest growing food-producing sector globally and now supplies about half of the fish consumed by humans (Anonymous 2003)[2]¹. As for all intensive culture of living organisms, maintenance of good health and the prevention and control of diseases constitutes a major challenge for cost-effectiveness and further sustainable development of the production. In current intensive salmonid farming, the feed represents the largest single cost. Production losses due to disease can substantially reduce profit margins. In the Norwegian salmon farming industry, a number of endemic viral infections and salmon lice (*Lepeophtheirus salmonis*) infestations represent still unresolved challenges to health and welfare of the farmed populations². In addition to zoo-sanitary strategies and the use of veterinary medicinal products such as vaccines and therapeutics, the use of tailored nutrient formulations and feed additives – so-called functional feeds – to boost disease resistance and/ or aid recovery after disease outbreaks has gained increased attention, and to a certain degree also application. Judged by recent fish farming magazine advertisements, a substantial proportion of the total feed input for sea farmed salmon currently constitute so-called functional feed formulations.

A common situation in controlled trials to demonstrate health effects of functional feeds is the application of the various test rations to groups (tank or pen populations), while measurement of the outcomes are performed on individual animals. This and other aspects have spurred discussions in the FHF committee «Robust fish and sustainable aquaculture», and a wish on the part of the FHF to organize a critical scientific assessment and -appraisal of the methods used in these kinds of experiments. The aims of the current work were therefore to provide:

1. *A scientific overview of published information on the effect of feeds or specific feed ingredients on the health of farmed fish.*
2. *A critical assessment of experimental designs, statistical and biomedical methods used in these studies.*
3. *Recommendations on study setups and statistical methods to document health effects of functional fish feeds.*

¹ <http://www.fao.org/docrep/013/i1820e/i1820e00.htm>

² <http://www.vetinst.no/Forskning/Publikasjoner/Fiskehelse rapporten/Fiskehelse rapporten-2011>

Material and working methods

The following delineations of the study subject were agreed with the project sponsor before commencement of the work:

- Data collection and assessment will comprise studies of or claimed outcomes relating to the effect of functional feeds and clinical diets on the clinical health aspects of farmed fish, whereas studies solely addressing other outcome parameters are excluded; e.g. growth, flesh quality or other production traits.
- In addition, the assessment will primarily concern studies of positive health effects, and not focus on the consequences of nutritional deficiencies or negative health effects of feeds or specific feed ingredients. Studies of certain ingredient's capacity to remedy negative health effects induced by other feedstuffs (e.g. soybean enteropathy) are nevertheless considered relevant.
- Publications of relevance to intensive production of salmonids are central to the sponsor's interest, but relevant examples and publications from other farmed species and from more extensive production systems may be addressed.

The work commenced with a collection of accessible literature aiming to capture both refereed scientific publications and so-called "grey literature" (industry reports and others) . Searches on the relevant keywords were performed in the databases available (ISI Web of Science, Biological Abstracts), and Google Scholar. Further searches were done in online databases of highly relevant publishers (ScienceDirect, Wiley Online, SpringerLink, Ovid) and in the project group's own literature files. Following the literature collection, which also encompassed a working seminar with a reference group of experts from academic and contract research institutions as well as the fish feed industry, the references were categorized according to species concerned and the outcomes measured. The majority of the initial reference collection dealt with experiments and trials conducted with non-salmonid aquatic species or were review articles, leaving in the end some 240 papers relating to salmonid species for scrutiny.

Based on detailed analysis of these selected parts of the collected literature, three working meetings were held among a sub-group of the authors to critically scrutinize the health

indicators, the study designs and the statistical and biomedical methods employed. The main contextual points and conclusions from this appraisal were discussed during a second meeting with the project reference group. The current draft report has been submitted to the steering committee and reference group for comments, and to the project's steering committee for adoption. In parallel, a separate popular non-technical summary in Norwegian has been produced to serve as a source of information for interested readers and decision-makers in the aquaculture industry.

Terminology and definitions

The current report encompasses various dietary interventions that can benefit fish health and modulate the outcome of clinical disease. This includes “**functional feeds**” as they apply to feed additives that have putative health benefits or can alleviate feed-associated pathologies. In addition, fish feed producers have developed so-called “**clinical diets**” or “**therapeutic diets**”, i.e. feed formulations tailored to meet altered nutrient requirements or take into account pathophysiological conditions that are caused by various diseases. These diets are developed to reduce pathology during disease, and support restitution and/or medicinal treatment.

Among the ingredients (additives or supplements) used in functional feeds are immunostimulants as well as pro- and prebiotics (see below). They are often used for prophylaxis (preventive measures) and fed to fish to prime their immune system and/or promote colonization of health-promoting intestinal microflora when the risk for infectious disease is considered elevated. So-called priming with functional feeds is typically recommended as a preventative strategy before seasonal- or developmental stage-dependent disease outbreaks or when disease occurrence at neighbouring sites increase the risk of water-borne contagions. They may, however, also have application during a disease outbreak.

On the other hand, clinical or therapeutic diets are modulated either in their nutrient concentration(s), the chemical form in which nutrients are present, and/or by supplementation with “nutriceuticals” - nutrients or other substances that may become conditionally essential and therefore required at higher levels during stress, disease or reconstitution. Various clinical diet formulations for different disease conditions are well-established in the pet food industry. For example, animals with kidney failure have reduced capacity to remove nitrogenous wastes and

certain minerals from the body. Therefore, diets for these patients contain reduced protein, salt and other mineral levels to prevent intoxications. Another example is cardiovascular disease in which diets with increased omega-3 fatty acid levels and reduced salt, saturated fats and cholesterol levels are beneficial. Patients with ailments of the exocrine pancreas (pancreatitis, pancreatic cancer etc.) produce little or no endogenous levels of the important digestive enzymes (proteases, amylase, lipases etc.) produced by this organ. Therefore, diets should contain hydrolysed (“pre-digested”) or purified sources of amino acids, sugars and fatty acids to prevent malnutrition.

In any case, collecting data to support the efficacy of such diets in controlled challenge or field trials can be difficult since critically ill fish may have severely decreased feed intake. Such trials may therefore require a different scope compared to trials in which the fish are primed prior to disease challenge.

The specific diet formulations developed by the various fish feed producers are generally proprietary and therefore difficult to specifically assess within the context of this report. Feed companies are restricted by EU legislation in marketing these products with any claims of health benefits, as is currently the case for feed additives generally.

Immunostimulants would in the term’s broadest sense comprise both nutritional and medicinal substances that are able to activate or stimulate one or more components of the specific or non-specific immune system. In the context of this report, however, vaccines and most therapeutants fall outside the scope and we will only deal with other substances, often derived from microbes, that possess an immunostimulatory effect when administered orally. In order to warrant characterization as a **primary immunostimulant**, we believe that a substance should be able to exert an immune-enhancing activity directly, without dependence on the intestinal microbiota (see below).

Probiotics are defined as live microbes used as feed supplements directly and that beneficially affect the host animal by improving its intestinal microbial balance (Fuller 1989)[4], and most importantly, providing a health benefit to the host (Anonymous, 2001) [1]³. Some authors have also extended this term to encompass preparations or components from inactivated cells that have also been reported to be beneficial to the host’s health (Salminen et al. 1999) [12].

³ http://www.who.int/entity/foodsafety/publications/fs_management/en/probiotics.pdf.

Authoritative literature exists on the evaluation of probiotics (FAO/WHO 2002)[13]. For most probiotics, the beneficial health effects are related to the microorganism's ability to out-compete or reduce viability of pathogenic microbes and positively influence the intestinal environment and epithelial cells, in mammals typically in the large intestine. Many if not most of the probiotic strains that have been evaluated in fish are derived from what is considered "normal" piscine intestinal microbiota.

Prebiotics, defined as non-digestible feed components that are metabolized by and stimulate the growth of intestinal microorganisms that are beneficial to the health of the host. Their benefits were first proposed by Gibson and Roberfroid 1995[7] and an initial review of their use in humans was provided by Manning and Gibson 2004[9]. Gibson et al. 2004[6] and finally Roberfroid 2007[11] later suggested the following re-definition: "*A prebiotic is a selectively fermentable ingredient that allows specific changes, both in the composition and/or activity of the gastrointestinal microflora that confers benefits upon the host well-being and health.*" According to the same author, prebiotics predominantly belong to the fructo-oligosaccharides (sometimes also called soluble fibres) and only oligo-fructose and inulin are believed strictly to meet this definition. However, other substance such as galacto-oligosaccharides, lactulose, mannan oligosaccharides, xylo-oligosaccharides, ismalto-oligosaccharides and other substance derived also from various soluble fibres are often also assigned to this group⁴. As opposed to probiotics, the prebiotics exert their health effects indirectly via their ability to modulate the host's gastrointestinal flora by providing fermentable substrate.

For this report, we use the term **controlled trial** for any study where experimental conditions may be controlled – in separate tanks or by other means securing detailed control of environment and husbandry, and ensuring that natural infectious patterns are not dominant. The term **field trial** is used to describe trials to study disease parameters that occur spontaneously in a natural setting, where natural environmental parameters prevail and husbandry generally is according to common industrial practice.

⁴ <http://en.wikipedia.org/wiki/Prebiotics>

Overview of the published literature

Review papers

There is a striking abundance of review papers and overview publications pertaining to various categories of health-promoting and/or immunostimulating fish feed ingredients or additives. Among the various categories, the richest review literature deals with probiotics, followed by beta-glucans and other immunostimulants (Table 1).

Table 1. Overview of the review literature on health promoting substances in fish feeds

<i>Substance category</i>	<i># of papers</i>	<i>Reference list #</i>
General nutrition	11	15-25
Beta-glucans, MOS etc.	7	26-31;82
Herbs	5	32-36
Immunostimulants general	14	37-50
Lipids	2	51-52
Nucleotides	2	53-54
Prebiotics	10	55-64
Probiotics	24	55;60;65-86
Vitamins and carotenoids	2	87-88

The large number of review articles compared to research articles may reflect popular trends among scientific institutions and funding bodies, but also the complexity in conducting and interpreting often confusing data from experiments and feeding trials. Contradictory data between experiments and trials have also been reported. Thus reviews may be written in an attempt to clarify, explain and recommend improved approaches, designs and analyses.

In subsequent tables, note that an attempt is made to give an indication of the number of cited articles that supply only indirect outcomes (most often immune parameters) versus those reporting clinical outcomes following challenge.

Cell wall polysaccharides (beta-glucans, mannan oligosaccharides)

Beta-glucans and other cell wall (cytoskeleton) polysaccharides are among the most studied immunostimulatory compounds in human and veterinary biomedicine. Health promoting products falling into this category may be derived from yeasts, algae, grains (barley) or mushrooms, and may undergo variable degrees of purification and processing before use. They may be in particulate or water-soluble forms, having some bearing on administration options. One important mode of action is that many of these substances are recognized as PAMPs (pathogen-associated molecular patterns) by the body's immune cells with pathogen recognition receptors, which then respond with increased activity, systemic recruitment, or proliferation. Among more than 100 articles dealing with their biological effects in aquatic organisms, there was, however, a clear majority of publications addressing only *in vitro* effects or reporting *in vivo* effects in non-salmonid species including crustaceans (not cited here).

Studies reporting effects of yeast cell wall beta-glucans and mannan oligosaccharides (MOS) have been frequently reviewed and their ability to stimulate various parts of the fish immune system is well established both *in vitro* and after *in vivo* injection, immersion or oral administration (Table 1).

We found 28 original papers reporting the possible health related effects of beta-glucans after oral administration to salmonids, of which 8 provided results from Atlantic salmon and 18 from rainbow trout. Nineteen of these papers reported clinical or disease-related outcomes (Table 2), while indirect health parameters (mainly non-specific immune functions) were reported in 17 papers. Only one of the articles (Refstie et al. 2010)[102] provides clinical data from an Atlantic salmon farming field situation.

Table 2. Overview of papers presenting data from in-feed glucans or mannan oligosaccharides (MOS) preparations

<i>Species</i>	<i>Indirect outcomes</i>	<i>Clinical outcomes</i>	<i>Reference list #</i>
<i>Atlantic salmon</i>	5	5	89-91;93;102-103; 134, 137
<i>Rainbow trout</i>	12	12	92; 94;96-99;104-113;204
<i>Other salmonids</i>	0	2	100-101

The first paper to report clinical effects of orally administered yeast glucans in Atlantic salmon was Raa et al. 1992[103] who reported that smolts that had received feed supplemented with one gram/kg of a particulate β -1,3/1,6 glucan derived from baker's yeast (*Saccharomyces cerevisiae*) for 70 days experienced approximately 50% reduced cumulative mortality compared to the control group after sea transfer and waterborne infection with *Vibrio salmonicida*. Five weeks of priming with this glucan preparation was also claimed to induce strong protection against experimental *Vibrio anguillarum* infection in pre-smolts. In the same paper, priming with beta-glucan supplemented feed was found to improve the effect of *V. salmonicida* vaccination, giving only 10% cumulative mortality after experimental challenge compared to 44% in the vaccinated group receiving feed without the glucan preparation. While the reported results are interesting, this conference proceedings paper lacks description of the materials and methods and study designs used, which strongly limits the reader's ability to critically scrutinise the data.

Nikl et al. 1993a,b[100-101] fed three concentrations (0,001, 0,1 and 1%) of a commercially available beta-1,3 glucan feed additive to juvenile Chinook salmon (*Oncorhynchus tshawytscha*) for 7 days, and subsequently subjected the fish in 3 replicates to bath challenge with the furunculosis bacterium *Aeromonas salmonicida* subsp. *salmonicida*. As compared to 16.7% cumulative mortality among controls, the groups that had received 0.1 or 1% glucan experienced a significantly lower cumulative mortality (1.7 and 5.8%, respectively). The glucan feed supplementation did not significantly potentiate the effect of immersion vaccination against furunculosis, that remained ineffective.

In a study to evaluate the prophylactic effects of a β -1,3 glucan derived from the macro algae *Laminaria hyperborea*, Dalmo et al. 1998[91] found that oral administration of this preparation for 10 days failed to protect Atlantic salmon pre-smolts against severe furunculosis challenge (waterborne infection in triplicate tanks) and that the reduction in mortality observed after cold water vibriosis (*V. salmonicida*) injection challenge was small and non-significant. This was in contrast to the earlier reports by Nikl et al. cited above, and Dalmo et co-workers attributed this discrepancy to the challenge conditions, with 87% mortality in the control group of their study as opposed to 17% mortality reported by Nikl et al. in 1993[100-101].

In a Tasmanian study, feeds supplemented with three beta-glucan preparations were administered for 7 days before the fish was exposed to a *Neoparamoeba sp.* protozoan to induce amoebic gill disease (Bridle et al. 2005)[89]. The experimental feeds failed to provide any protection against the infection, or any effect of respiratory burst activity of head kidney macrophages, despite salmon macrophages responding significantly when exposed to the glucan preparations *in vitro*. This outcome was confirmed by Zilberg et al. 2000[93] who tested oral priming of seawater reared salmon with a commercial beta-glucan preparation (1 g/kg feed) for 21 days, followed by cohabitant exposure of the fish with amoeba beginning 10 days later. Oral supplementation with levamisole, an anthelmintic that also has immunomodulating effects also failed to provide any protective priming effect, while this chemotherapeutant appeared to assist in the control of AGD when administered into freshwater bath treatment.

In a recent field trial, groups of 200-gram Atlantic salmon kept in sea cages were fed diets containing 32% of soybean meal or 14% soybean meal plus 14% sunflower meal, with or without supplementation with a processed β -1,3/1,6 glucan or a mannan oligosaccharide (MOS) rich feed ingredient, for 70 days (Refstie et al. 2010)[102]. Groups kept on the experimental diets containing soybean meal developed soybean enteritis symptoms during the trial as compared to a fishmeal control group, but the severity was reduced in fish receiving diets with only 14% SBM inclusion. At the end of the feeding period, the salmon receiving the feed with soybean meal + sunflower meal mix showed a reduced sea lice burden, which was further reduced in the glucan supplemented group but not in the MOS supplemented group. The authors conclude that beta-glucan strengthened the salmon lice preventing effect of the dietary sunflower meal, whereas no significant effect of glucan or MOS was seen on the severity of the soybean enteritis.

In rainbow trout, studies reporting direct measures of health such as survival following infection after feeding of glucan-containing products were all performed in freshwater rearing systems. Siwicki et al. 1994[107] found slight to moderate (~40%) reduction in post-challenge *Aeromonas salmonicida* mortality after orally administering six different immunostimulants (including beta-glucan and dried yeast) for 7 days to 200-gram rainbow trout. Jeney et al. 1997[97] obtained inconclusive results when experiencing a spontaneous *Flexibacterium columnaris* outbreak during a stress experiment with glucan fed rainbow trout post-smolts, while there were some minor responses in a number of immune parameters. In an experiment where 90-100 gram rainbow trout were given β -1,3/1,6 glucan supplemented feed prior to yersiniosis vaccination,

Siwicki et al. 2004[106] reported increased specific immune responses as compared to fish receiving control feed. No challenge data were, however, reported in this paper. Also Staykov et al. 2007[108] found an approximately 50% reduction in low overall mortality of 30- and 100-gram rainbow trout during feeding for 42 days with a mannan oligosaccharide (MOS) supplemented feed under normal rearing condition. No challenge occurred in this field study. Finally, Sealey et al. 2007[105] observed that 15-gram rainbow trout that were given feed supplemented with partially autolysed yeast or a proprietary probiotic for 9 weeks showed increased survival over control fish (61-64% RPS) after IHN virus challenge by inoculation. Using the same challenge model in a second experiment, this group (Sealey et al. 2008)[104] further demonstrated that a proprietary β -1,3/1,6 glucan feed ingredient as well as replacement of the wheat in the feed formula with glucan-rich barley flour was able to increase the IHN survival of 15-gram rainbow trout. Preventive effects of beta-glucans on ectoparasite infestations of rainbow trout have recently been reported by two other groups. Three weeks of feeding 20-30 gram rainbow trout with a beta-glucan feed additive was shown to reduce the gill pathology after oral *Loma salmonae* challenge by approximately 50% (Guselle et al. 2010)[96]. A clear reduction in *Ichthyophthirius multifiliis* settlement was reported after feeding a beta-glucan-supplemented diet for 35 days by Lauridsen and Buchmann 2010[98].

While the beta-glucan literature comprises many controlled *in vivo* studies documenting both immune stimulatory effects and increased survival following challenge to experimental infections, only a small proportion of the studies demonstrate clinical effects in the field or in semi-industrial rearing situations. Given the widespread use of beta-glucans and MOS preparations in fish feeds over many years, this is surprising.

Other immunostimulatory ingredients

In addition to the cell wall polysaccharides, several ingredients or chemical substances with known immuno-stimulatory properties (known from mammalian medicine or from *in vitro* experiments with fish immune cells) have been tested in live fish. Excluding the nucleotide category (see below), we have identified some 20 papers contributing original data from in-feed studies in salmonids. The range of ingredients is quite variable, from well-defined nutrients (e.g. betaine, astaxanthin and other carotenoids), via extracted alginates, bovine lactoferrin, lysozyme, bacterial LPS and antibacterial peptides, to a range of herbal preparations inspired from artisanal

medicine. Nearly half of these publications report clinical outcomes such as survival or pathology following challenge, but only two of the clinical outcome studies involve Atlantic salmon (Table 3).

Table 3. Overview of papers presenting data from in-feed use of immunostimulatory ingredients other than glucans or nucleotides

<i>Species</i>	<i>Immune parameters</i>	<i>Clinical outcomes</i>	<i>Reference list #</i>
<i>Atlantic salmon</i>	2	2	118-119
<i>Rainbow trout</i>	13	6	114-117; 120-130; 132
<i>Other salmonids</i>	0	1	131

Most of these classes of immunostimulatory and health promoting substances are relatively well discussed in the general immunostimulant review literature (Azad et al. 2007[39], Gallindo-Villegas and Hosokawa 2004[42], Raa 2001[46] but a recent upsurge of interest can be noted in the review of studies involving medicinal herbs (Chakraborty & Hancz 2011[32], Harikrishnan et al. 2011[35], Chitarasu 2010[33]; Jeney et al. 2009[36].

Muona and Virtanen 1995[119] were unable to report any clinical benefits to *Vibrio anguillarum* challenge after feeding Atlantic salmon glycine betaine. No or only minor clinical protection was observed after feeding rainbow trout fry or fingerlings a commercial alginate preparation before experimental *Flavobacterium columnare* challenge (Suomalainen et al. 2009)[130].

Among the studies with inclusion of herbal substances, 14 days of feeding the garlic bioactive component allicin to rainbow trout increased the *in vitro* bactericidal activity of serum and was able to protect the fish from *Aeromonas hydrophila* challenge (Nya and Austin 2009; Nya et al. 2010)[120;123]. As the experimental infection of this study was performed by inoculation, however, it is difficult to extrapolate the results to a practical farming situation.

Oral administration of bacterial lipopolysaccharide derived from *Aeromonas salmonicida* was tested for its immunostimulatory properties by Guttvik et al. 2002[118] when fed to Atlantic salmon fry. Following 62 days of feeding LPS-supplemented diets at two levels of inclusion, the cumulative mortality following experimental *A. salmonicida* bath challenge was well in excess

of the mortality in the control fed group. The outcome was confirmed in a repeated setup where differences between LPS-primed and control fed groups were small and statistically non-significant in response to experimental challenges with either *A. salmonicida* or *V. anguillarum*. In contrast, Nya & Austin 2010[122] reported that LPS purified from *E. coli* endotoxin, when fed to 10-gram rainbow trout for 14 days (four levels of inclusion), provided protection when tested in their *A. hydrophila* inoculation challenge model.

In general, the literature on the non-glucan immunostimulatory feed supplements shows a striking paucity of confirmatory reports from clinical studies in field-like situations.

Lipids, bile salts, organic acid salts, and others

The research on specific health effects in salmonid fish of in-feed lipids, bile salts, organic acid salts or urea is quite scarce and comprises less than 20 papers. Unlike other areas of the health feeds literature, most have been focusing on clinical outcomes and in particular on the alleviation of intestinal pathology induced by soybean derived ingredients (Table 4).

Table 4. Overview of papers presenting investigations on health effects of lipid, bile salts, urea etc. in salmonid feeds

<i>Species</i>	<i>Immune parameters</i>	<i>Clinical outcomes</i>	<i>Reference list #</i>
<i>Atlantic salmon</i>	2	4	139-141; 143; 150-151
<i>Rainbow trout</i>	1	9	142; 144-146; 149; 152-156
<i>Other salmonids</i>	0	2	147-148

Several field trial papers show clear support for positive clinical effects of supplementing Atlantic salmon diets with a saturated fatty acid, tetra-decylthioacetic acid (TTA). Substantial reduction in mortality was found during outbreaks of the viral diseases heart and skeletal muscle inflammation (HSMI; Alne et al. 2009)[139] and infectious pancreatic necrosis (IPN; Rørvik et al. 2007)[150]. Omega-3 dietary supplementation was shown to reduce mortality during outbreaks of the bacterial infections furunculosis and cold water vibriosis (Rørvik et al. 2003)[151]. Other fatty ingredients have been repeatedly shown to reduce tissue micro pathologies in Atlantic salmon and rainbow trout (Iwashita et al. 2008[144]; Suzuki and

Yamamoto 2004[153], Bell et al 1993[140]), or to improve immune parameters (Gjøen et al. 2007[143], Kiron et al. 1993[146], Erdal et al. 1991[143]) yielding support for indirect health effects.

Bile salt supplementation has been shown by Japanese workers to have some beneficial effects on intestinal pathologies caused by soybean meal in rainbow trout, but to our knowledge this has not been confirmed in Atlantic salmon. A first report of beneficial effects of urea supplemented feeds on winter ulcers in rainbow trout (Rørvik et al 2000)[152] has to our knowledge not been repeated. It should also be noted that supplementation of plant-based rainbow trout diets with organic acid salts did not produce significant differences in intestinal morphology (Gao et al. 2011)[142].

Nucleotides

The mechanisms of action of nucleotides include provision of components of cellular energy (e.g. AMP) as well as cellular replication and protein synthesis (DNA and RNA). Based on this, they appear to have a dual function, both as immunostimulants and in tissue restitution during and following disease. The list of scientific literature on clinical effects of nucleotide supplemented diets in salmonids comprises only six papers, while eight further papers (not cited here) deal with health effects in non-salmonid species. The first and dominant publications are by Burrels et al. 2001 a,b [134-135] who covered the three dominant salmonid farming species world-wide (Atlantic salmon, rainbow trout and Coho salmon), challenged with selected bacterial, viral and parasitic infections of prime relevance to commercial salmonid farming.

The first of these papers (Burrels et al. 2001a)[134] reports the outcome of four separate experimental infection trials carried out in Scotland, Norway and Chile with a proprietary feed supplement containing a mixture of cytosine- (CMP), uridine- (UMP), adenosine- (AMP) and guanidine (GMP) monophosphate esters and RNA. After 2 or 3 weeks, representatives from the dietary groups were subjected to experimental challenge with *Vibrio anguillarum* (trial 1), infectious salmon anaemia virus (trial 2), *Piscirickettsia salmonis* (trial 3) or sea lice copepods (trial 4).

The second paper (Burrels et al. 2001b)[135] reports the effect of in-feed dietary nucleotides on furunculosis vaccine protection, a number of physiological parameters, and growth in Atlantic

salmon smolts immediately prior to or following seawater transfer, as observed in five distinct trials. The study setups and clinical outcomes are summarized in Table 5, indicating clear beneficial health effects from nucleotide dietary supplementation, as well as from β -1,3/1,6 glucan in one group that was included in the vibriosis challenge experiment.

Table 5. Summary of nucleotide trial setups and outcomes from Burrels et al. 2001 a,b[134;135]. Inclusion level of the proprietary nucleotide supplement was 2 g/kg feed (0.2%). FW= fresh water; SW= sea water.

Species	Priming period	Water quality	Challenge			Results
			Organism	Route of exposure	# of replicates per treatment	
RT	3 weeks	FW	<i>V. anguillarum</i>	Bath	4 (tagged & mixed)	Control mortality: 49% Nucleotide fed group: 31% Glucan fed group: 43%
AS	2 weeks	SW	<i>ISA virus</i>	Cohabitant	3	Control mortality: 48% Nucleotide fed group: 36%
CS	3 weeks	SW	<i>P. salmonis</i>	i.p.	3	Control mortality: 77% Nucleotide fed group: 47%
AS	3 weeks	SW	<i>L. salmonis</i>	Bath	4 (tagged & mixed)	Control group: 2.5 copepodid/fish Nucleotide fed group: 1.4 copepodid/fish
AS	3 weeks + 5 weeks post -vacc	FW	<i>A. salmonicida</i>	Cohabitant	3 + 3 unvaccinated	Control (unvaccinated) mortality: 38% Control group (vaccinated): 6% Nucleotide fed group (vaccinated): 2%
			none	None	3	Control group vaccinated: Ab titre 1:144 Nucleotide fed group vaccinated: 1:60

The referred papers are relatively unique in the literature concerning health effects of functional feeds in that they report experiments carried out in seawater reared salmon and trout, as opposed to the vast majority of articles dealing with freshwater reared rainbow trout. In our opinion these studies, including the challenge methods, allocation of groups to holding units (tanks) and the use of replicates, may also serve as examples of high-quality controlled trials with robust and relevant outcome assessments that allows at least some degree of extrapolation to practical farming situation. However, we suspect that the statistical methods used (ANOVA) may have rendered the outcome analysis insensitive, producing p values that might have led the authors to under-rate the outcomes as non-significant. Only a re-analysis of the original data behind can answer this question. A recent trial performed with a proprietary functional feed containing an unidentified amount of nucleotide supplement (Tacchi et al. 2011)[137] given over 16 weeks indirectly revealed putative health effects as indicated by distinct transcriptome profiles. However, no clinical outcomes were reported.

Further papers on health effects of nucleotide enriched diets are from experiments in rainbow trout. Adamek et al. 1996[133] were the first to report results from nucleotide feed supplementation to fish. During a 35-day feeding trial where no natural challenge was evident, all groups (including controls) showed 98-100% survival. Leonardi et al. 2003[136] reported data from a trial with 80-100 gram rainbow trout fed a nucleotide-rich diet for 120 days and subsequently challenged with IPN virus by intra-peritoneal injection. While all the fish receiving control feed died within eight days following inoculation, all of the nucleotide-fed fish survived through this date. The authors further found that leucocytes of nucleotide-primed fish responded stronger during *in vitro* proliferation assays. The validity of the results for practical rainbow trout farming may, however, be questioned both due to the challenge route (i.p. inoculation) and the extremely low number of animals (n=8) per diet group.

Finally, Tahmasebi-Kohyani et al. 2011[138] recently reported a rather comprehensive assessment of dietary nucleotide effects in freshwater reared rainbow trout, where 3 replicate groups of 23-gram fish received diets supplemented with 0 to 2.0 g/kg nucleotide. Besides the assessment of non-specific immune functions that showed dose-dependent elevations in the nucleotide supplemented groups, an intra-peritoneal challenge with virulent *Streptococcus iniae* was carried out in 3 replicates á 10 fish per dietary group. Compared to only 16% survival in

control fish, survival in the nucleotide supplemented groups was dose-dependent, reaching 62% in the fish receiving 2% nucleotides in the feed. Except for the experimental infection route that admittedly remains artificial, the clinical setup of this trial has a good design that supports the relevance of its outcomes for freshwater trout farming in temperate to warm inland waters.

Prebiotics

As opposed to numerous articles reporting data from the use of prebiotics in humans and mammals, there are few papers on the effects of inulin supplementation of salmonid diets (Table 6). Interestingly, two reports indicate adverse rather than beneficial effects. Olsen et al. 2001[160] were the first to report that exchange of 15% dextrin with 15% inulin in a casein-based diet caused vacuolizing degeneration to the enterocytes of Arctic charr (*Salvelinus alpinus*) after 4 weeks of feeding. In a subsequent paper, these changes caused by the same inulin supplement was suggested to coincide with a decreased level of adherent bacteria in the hindgut (Ringø et al. 2006)[161], which confirmed the ability of inulin to modulate the intestinal flora. The authors concluded that unlike reported data in rats, this level of dietary inulin could be too high in carnivorous salmonids. Testing a 7.5% inulin-supplemented diet in Atlantic salmon post-smolts, no inflammatory changes and only a small increase in vacuolization in the distal intestine was seen after 3 weeks of feeding, as opposed to marked morphological changes in a group receiving 25% soybean meal supplementation (Bakke McKellep et al. 2007)[157]. Grisdale-Helland et al. 2008[95] reported small, statistically non-significant reduction in serum lysozyme activity and in oxygen radical production in blood neutrophilic granulocytes in Atlantic salmon fed prebiotics or MOS for 4 months. The recent prebiotic literature in salmonids also comprises a paper by Kristiansen et al. 2011[159], who reported that 15 weeks of feeding a proprietary prebiotic of undisclosed content gave a similar *in vivo* density of attached bacteria as control fed fish. No further clinical results were reported in this study.

Thus all of the prebiotic studies done in salmonids are devoid of data regarding effects on susceptibility to infectious disease. Whether the preventive effects of dead probiotic cells against furunculosis (Irianto and Austin 2003)[158] or reduction of soy enteritis by adding a bacterial meal based on *Methylococcus capsulatus* (Romarheim et al. 2001)[162] should be attributed to prebiotic or probiotic effects may be dependent on definition. However, these reports indeed represent rare health promoting findings among the prebiotic literature in salmonids.

Table 6. Overview of papers presenting investigations on health effects of prebiotics in salmonid feeds

<i>Species</i>	<i>Immune parameters</i>	<i>Clinical outcomes</i>	<i>Reference list #</i>
<i>Atlantic salmon</i>	<i>2</i>	<i>4</i>	157; 159; 162-163
<i>Rainbow trout</i>	<i>1</i>	<i>1</i>	158
<i>Other salmonids</i>	<i>1</i>	<i>1</i>	160-161

Probiotics

Abundant literature exists on the direct and indirect health effects of live microbial feed supplements, especially taking into consideration the still limited use of probiotic feed additives in industrialised salmonid production. High-temperature extrusion and drying, which constitutes the predominant technology in salmonid feed manufacture, obviously presents major obstacles to the inclusion of live microorganisms to current formulated feeds. It therefore seems fair to postulate that any large-scale industrial utilization of in-feed probiotics for salmonids will be dependent on novel feed producing technologies that can avoid the inactivation of beneficial microbial additives while retaining adequate heat treatment of raw materials, and formation of pellets ensuring minimal leakage of nutrients and suitable for automated feed distribution. Among some 50 papers, studies performed in rainbow trout dominate the literature on health effects of probiotic microorganisms for use in salmonid farming. A relatively wide range of potential probiotic bacteria have been tested, whereby results from studies involving lactic acid bacteria, *Bacillus* spp. or yeast supplementation of feeds are the most frequently reported (Table 7).

Table 7. Overview of papers reporting indirect or clinical health effects of supplementing salmonid feeds with various categories of probiotic microorganisms

Group of microorganisms	Indirect outcomes	Clinical outcomes	References #
<i>Aeromonas</i> and <i>Pseudomonas</i>	3	8	164-170; 205-207
<i>Bacillus</i> spp.	1	9	165-166; 168; 171-176; 198
Enterococci and micrococci	2	3	178-183
Lactic acid bacteria	12	13	167; 177; 184- 203, 207
Yeasts	1	9	209-217

Aeromonas* and *Pseudomonas

The use of *Aeromonas hydrophila*, *Vibrio fluvialis* and *Carnobacterium* strains for probiotic purposes was first reported by Irianto and Austin 2002b[167], who found that 14-days of feeding diets supplemented with these strains provide good protection of 12-gram rainbow trout juveniles challenged with furunculosis by i.p. injection as well as by waterborne exposure. Brunt and Austin 2005[169] identified an *Aeromonas sobria* strain highly effective in preventing clinical disease in rainbow trout caused by i.p injection of rainbow trout with *Lactococcus garviae* or *Streptococcus iniae*. The *A. sobria* strain as well as a *Brochotrix thermospacta* strain was also found to be effective in preventing cutaneous ulcers following inoculation with *Aeromonas bestiarum* and following waterborne infection with *Ichthyophthirius multifiliis* in rainbow trout (Pieters et al. 2008)[164]. Finally, oral administration of *A. sobria* provided good protection to rainbow trout against experimental *V. harveyi* infection, while a putative probiotic strain of *A. salmonicida* gave no significant effect (Arijo et al. 2008)[170].

We have found no papers on the use of putatively probiotic *Pseudomonas* strains for feed supplementation, but Spanggaard et al. 2001[207] reported six strains, which were antagonistic to *V. anguillarum* *in vitro*, provided only faintly reduced mortality (13-43%) when administered as a bath prior to immersion challenge of 20-gram rainbow trout. However, bath administration of an *in vitro* antagonistic *Pseudomonas fluorescens* strain failed to provide protection in a furunculosis (*A. salmonicida* subsp. *salmonicida*) cohabitation challenge experiment in Atlantic salmon parr (Gram et al. 2001)[205].

Bacillus spp.

Raida et al. 2003[176] reported a small-scale experiment in which 4- month old rainbow trout given feed supplemented with a proprietary mixture of two probiotic *Bacillus* spp. for 42 days gained moderate protection (approximately 35% RPS) against intra-peritoneal *Yersinia ruckeri* challenge. Independently, a Scottish research group (Brunt and Austin 2005 [169]; Brunt et al. 2007[166]) reported that rainbow trout given probiotic *Bacillus* spp. or *A. sobria* cultures showed a strong reduction in mortalities (0-13% vs. 80-100% in control fed fish) after experimental infection with *Streptococcus iniae*, *Lactococcus garviae*, *A. salmonicida*, *V. ordalii*, *V. anguillarum* or *Y. ruckeri*. Protection was evident in both waterborne and inoculation challenge experiments. In a follow-up experiment, the protection conferred by these probiotic strains against yersiniosis was attributed to components that could be extracted from inactivated bacteria (Abbass et al. 2010)[168]. Similarly, high levels of protection were obtained in a separate trial using i.p. *A. salmonicida* challenge of rainbow trout after feeding *B. subtilis*-coated feed for 14 days (Newaj-Fyzul et al. 2007)[171]. Independent confirmation of the probiotic potential was reported by Capkin and Altinok 2009[172] who also demonstrated largely improved survival to immersion challenge with *Yersinia ruckeri* after supplementing rainbow trout feeds with a mixture of *B. mojavensis* and *Enterobacter cloacae* cultures. These effects of the probiotic *Bacillus* strains have, however, not been confirmed in larger-scale or field-type settings as yet.

Micrococci

Using *Vibrio anguillarum* and/or *V. ordalii* injection challenges, Sharifuzzaman and various co-authors [179;180;181] showed that a probiotic micrococcus belonging to the genus *Kocuria* would confer strong protection (16-30% vs. 70-90% in control groups) to rainbow trout after 2 weeks or longer oral administration. Using an inoculation protocol, the authors also attributed the protection to cell wall and whole cell proteins of the probiotic strains (Sharifuzzaman et al. 2011).

Lactic Acid Bacteria (LAB)

Gildberg et al. 1995[188] reported that Atlantic salmon fry given a diet supplemented with a LAB culture showed higher cumulative mortality after experimental furunculosis (*A. salmonicida* subsp. *salmonicida*) challenge than the control fed fry. In contrast, Nikoskelainen et al. 2001[192] reported that 30-gram rainbow trout fed *Lactobacillus rhamnosus* supplemented feeds showed protection against furunculosis challenge, but with an inconsistent dose-response pattern. In several challenge experiments involving rainbow trout fry, rainbow trout fingerlings or Atlantic salmon fingerlings, Robertson et al. 2000[177] found that a *Carnobacterium* strain when used as an in-feed probiotic for 14 days would increase the survival after *A. salmonicida*, *V. ordalii* and *Y. ruckeri* challenge. In two more recent studies reporting the outcome of experimental *Lactococcus garviae* infection in 25-30 gram rainbow trout, administration of LAB supplemented feeds for 21-30 days prior to challenge was able to increase the survival moderately, but not in all LAB-fed groups (Vendrell et al. 2008[201], Péres-Sánchez et al. 2011[200]). In all of these trials, the use of waterborne exposure represents a clear strength, while the study design must be criticized. In all cases, both feeding and challenge were performed in separate tanks without replicates, rendering the outcomes highly vulnerable to uncontrolled group effects and thus weakening the conclusive evidence of the results.

Yeasts

Barnes et al. 2006; 2010[210;213] found that addition of a proprietary yeast culture preparation to the feeds of rainbow trout fry and juveniles could reduce mortality during early rearing by approximately 50% compared to the control group (5.2 vs. 10.8%) in a dose-dependent manner, while only minor effects were seen beyond the 5-gram stage. The same authors reported similar effects in other salmonid species (Barnes et al. 2006[211], 2007[212], and their results have been independently confirmed by Zargham et al. 2011[217] and Tukmechi et al. 2011[216]. The latter group also reported that rainbow trout receiving feed supplemented with beta-mercaptoethanol treated *Saccharomyces cerevisiae* showed increased survival after i.p. challenge with *Y. ruckeri* (67% vs. 47% in controls), while the improvement seen with untreated or omega-3 supplemented yeast cultures was smaller and non-significant.

Further results were reported after dietary yeast supplementation in a 5-month trial using feed top-coated with *Saccharomyces cerevisiae*, LAB, or florfenicol during early rearing of rainbow trout (Aubin et al. 2005)[209]. The fish group initially given dietary antibiotics and the group

given continuous yeast supplemented feeds developed only low prevalence of vertebral column compressions (2-4%), while the prevalence of vertebral malformations in the control group was 13%. Initial short-term yeast supplementation or short-term or long-term LAB supplementation yielded only minor and non-significant improvements. The authors interpreted this finding as supportive evidence for the role of *Flavobacterium columnare* infection in the aetiology of this syndrome.

In summary, the beneficial effects reported in the probiotics literature should be validated in large-scale and field-like studies. This would also in particular extend to studies involving stressful procedures (with experimental or natural bacterial challenge). It is at such critical situations that the role of biotic dietary supplements is expected to offer additional support against infection and/or disease outbreaks.

Vitamins and carotenoids

The vitamin requirements of salmonids have been comprehensively researched and the results summarised in recent review articles by Waagbø 2010[88] and Verlhac Trichet 2010[23]. We found some 20 recent papers reporting effects of mainly vitamins A, C and E in rainbow trout or Atlantic salmon immune functions or clinical health, including four papers addressing the effect of carotenoids (Table 8).

Table 8. Overview of papers presenting data from in-feed use of vitamins to enhance immune responses or clinical health outcomes in salmonids

<i>Species</i>	<i>Immune parameters</i>	<i>Clinical outcomes</i>	<i>Reference list #</i>
<i>Atlantic salmon</i>	6	2	224-227; 232-233; 238
<i>Rainbow trout</i>	7	5	218-223; 228-231; 234-237

The need for vitamin supplementation of salmonid diets – particularly with vitamin E with its antioxidant properties - is closely associated with high lipid contents and the risk for oxidation during high-temperature extrusion, drying and/or storage. Barrows et al. 2008[222] thus concluded that supplementation beyond the standard recommendation (NRC 1993) might be

required in feeds for young, rapidly growing rainbow trout, and advocated adjustment of the supplementation to account for destruction of vitamins during the manufacturing process in order to ensure sufficient dietary supply. The dose-dependent ability of dietary vitamin C supplementation to prevent clinical signs of vitamin E deficiency in first feeding Atlantic salmon was reported by Hamre et al. 1997[225]. Vitamins C and E have complimentary antioxidative effects, working in different compartments – water and lipid phases, respectively. Vitamin C also has the ability to regenerate vitamin E. Therefore, simultaneous supplementation can be advantageous.

Vitamin boosting to promote health was attempted by Navarre and Halver 1989[230], who fed overdoses (up to 20 times) of vitamin C (ascorbic acid) to groups of young rainbow trout for 28 weeks before subjecting them to challenge with *Vibrio anguillarum* with or without prior vaccination. The un-immunised fish receiving feed containing 500 to 2000 mg ascorbic acid per kg were protected both against modest inoculation challenge and via immersion. All fish except the group that received un-supplemented feed developed good protection against the experimental infection within two weeks of immunization. Erdal et al. 1993[224], however, reported that mega-doses of dietary vitamin C or vitamin E fed for 10 weeks were unable to provide protection against *Vibrio salmonicida* in Atlantic salmon parr. In the same trial, vitamin C feed induced increased antibody responses after *Yersinia ruckeri* vaccination, while no beneficial effect was evident following experimental challenge with the same organism. In both cases, the challenge was performed by inoculation.

The most convincing clinical effects of vitamin boosting in salmonids were reported by Wahli et al. (1998, 2003). In the first paper [237], feeds supplemented with various doses and combinations of vitamin C+E were fed to 180-gram rainbow trout for 7-18 weeks before groups of fish were subjected to infection experiments. Groups fed one or both vitamins in high doses showed strong relative protection after viral haemorrhagic septicaemia (VHS) or *Yersinia ruckeri* challenge, while the highest mortalities were experienced in the vitamin deficient groups. The same pattern was evident in fish exposed to *Ichthyophthirius multifiliis*, although differences remained non-significant. An important strength of this paper is the comprehensive design and that all experimental infections were by bath. In 2003 [236], members of the same research group continued to demonstrate that supplementation of 1000 mg/kg ascorbic acid to the feeds

would significantly support the time course of cutaneous wound healing in rainbow trout as assessed by histological methods.

While three papers report improvement of salmonid immune functions by carotenoid feeding, only one out of eight tested carotenoids, a synthetic astaxanthin, was found to increase the survival of rainbow trout fry subjected to modest IHN virus challenge (Amar et al. 2012)[218]. However, as supplementation with the same concentration of other, chemically similar substances and challenges using 10-fold higher concentration of the virus bath gave statistically insignificant results, we feel that this outcome should be verified by further experiments.

Alleviation of feed induced intestinal pathologies

In the last decade, the importance of gut health in farmed fish has gained recognition in Norway. Various presumably non-infectious conditions affecting the gastrointestinal tract have been identified, such as gastric dilatation syndrome, gastric ulcers, lipid malabsorption syndrome (steatorrhea), intestinal obstruction (colic), feed ingredient (legume)-induced enteropathies, and intestinal tumours. Multiple factors appear to be involved in the aetiology of most of these digestive disorders/conditions, among which diet involvement is assumed or has been demonstrated for most. Hence changes in feed formulation, feed processing, contaminant levels and/or feeding practices may prevent, alleviate symptoms or aid in recovery. For soybean meal-induced enteropathy in salmonids, an inflammatory response in the distal intestine and by far the most well characterized of these conditions. Various feed additives such as cholesterol, bile salt and/or bacterial meal from *Methylococcus capsulatus* grown on natural gas appear to alleviate the severity of the inflammation. However, the underlying mechanisms are not well understood. Various processing measures of soybean meal (SBM) presently employed decrease activity/concentration of some detrimental anti-nutritional factors and/or other antigens relatively effectively. Extraction with aqueous ethanol solution to obtain soy protein concentrate (SPC) is apparently the most effective but also the most expensive method. Fermentation of soybean meal, for example by lactic acid bacteria (Refstie et al. 2005)[203] or mixture of thermophilic *Bacillus* sp. (Yamamoto et al. 2010)[175], has also been shown to effectively reduce the inflammatory response in rainbow trout, but is currently not considered a practical, cost-effective processing method.

Modulation of soya bean meal-induced enteropathy

Twibell and Wilson 2004[8] demonstrated that adding cholesterol to SBM-containing diets for juvenile channel catfish resulted in improved growth and feed intake. However, histological evaluation of the intestine was not carried out in this study. In more recent studies, supplementation of bovine bile salts or cholesterol to extracted SBM-based diets (Yamamoto et al., 2007)[156] as well as diets supplemented with the ethanol extract from defatted SBM (Yamamoto et al., 2008)[155] for rainbow trout was reported to ameliorate the inflammatory response in the distal intestine caused by the diets containing non-supplemented SBM and soybean ethanol extract (the by-product from processing used to produce SPC). Bile salt supplementation also restored growth, feed efficiency and intestinal maltase activity to comparable levels reported in the control group. This type of investigation has not been reported in Atlantic salmon. Cholytaurine was also reported to ameliorate the negative impact of soybean saponin and/or lectin on intestinal structure (Iwashita et al. 2008)[144]. Cholesterol and bile salts may bind amphipathic saponin and thus prevent any deleterious effects of these on the intestinal tissue.

Supplementation of a SBM-containing diet with 30% bacterial meal made from *Methylococcus capsulatus* grown on natural gas has recently been reported to prevent development of the inflammation caused by SBM (Romarheim et al., 2011)[162]. The authors speculated that high levels of nucleotides, phospholipids and small peptides in the bacterial meal may be beneficial for intestinal homeostasis and may thus prevent inflammation. Yet the effects of supplementing SBM-containing diets with lower levels of bacterial meal have not been reported and the mechanisms involved are not well understood. More research is needed to investigate this.

Summary assessment

As documented in the literature review, the two main clinically beneficial effects from any feed intervention on infectious diseases will be either or both of the following:

1. Priming of fish defence systems or intestinal microbiota to reduce the risk of contracting infection or clinical disease and hence reduce losses occurring on a site or in a pen, including limiting the spread within a pen or inside a location
2. Reducing the deleterious pathophysiological effects of disease, thus reducing losses and aiding in restitution once infection is established

Based upon the cited literature, the evidence for beneficial clinical effects derived from the various categories of putative health promoting feed additives and nutrients, we have tried to summarise our main findings in Table 9.

Table 9. Overall evaluation of published evidence for beneficial effects of various feed ingredients from controlled trials and field studies

Substance	Summary evaluation
Cell wall polysaccharides (beta-glucans, mannan oligosaccharides)	Beneficial clinical effects on bacterial, viral and ectoparasitic infections shown in controlled trials with rainbow trout. Limited effects on sea lice settlement shown in one Atlantic salmon field trial.
Other immunostimulants	Some support for beneficial clinical effects in controlled clinical trials in rainbow trout. No reports of clinical effects from the field.
Lipids, bile salts, organic acid salts, and others	Some support for improved survival or reduced gut pathology from field trials in Atlantic salmon and rainbow trout. No reports from controlled clinical trials.
Nucleotides	Beneficial clinical effects on bacterial, viral and ectoparasitic infections shown in controlled trials with Atlantic salmon and rainbow trout. No reports of clinical effects from field trials.
Prebiotics	No support for beneficial clinical effects from controlled clinical trials nor from field trials.
Probiotics	Lactic acid bacteria, Pseudomonas/Aeromonas and Micrococci: beneficial clinical effects on bacterial, viral and ectoparasitic infections shown in some controlled trials with rainbow trout. Yeasts: some support for increased survival from field trials.
Vitamins and carotenoids	Some support for beneficial clinical effects in controlled clinical trials in rainbow trout. No support from field trials.

Among the more than hundred published papers reviewed above, only a few deal with results obtained from commercial clinical diets or proprietary health promoting feed ingredients or additives (functional feeds) that are actively supplied to the Norwegian market (Table 10).

Table 10. Overview of published papers from farmed salmonids providing results suggesting beneficial clinical effects of branded feeds or ingredients being marketed in Norway

Proprietary brand	Papers reporting beneficial clinical effects
Macrogard (β-1.3/1.6 glucan)	Raa et al. 1992 [103] Refstie et al. 2010 [102] Sealey et al. 2008 [104]
ProVale (beta-glucan)	Guselle et al. 2010 [96]
Unbranded ingredient cocktail	Xueqin et al. 2012 [204]
Unbranded glucan	Lauridsen and Buchmann 2010 [98]
Ascogen/Optimun (nucleotide mixture)	Burrells et al. 2001 [134] Burrells et al. 2001 [135] Leonardi et al. 2003 [136] Tahmasebi-Koyani et al. 2011 [138]
Rovimix Stay-C (Vitamin C)	Wahli T et al. 1986 [235] Verlhaq et al. 1996 [234] Wahli, T et al. 1998 [237] Verlhaq et al. 1998 [110] Wahli et al. 2003 [236]
Carophyll	Amar et al. 2012 [218]

Effects of clinical diets on infectious diseases have hitherto mainly been demonstrated in controlled studies where the feeds have been evaluated for their capacity to minimize morbidity, mortality and clinical symptoms, reduce performance losses and enhance restitution once an infectious disease was established. Studies have been carried out using various experimental challenge techniques, and we consider such challenge studies relevant and valid especially where natural routes of infection (via the water) were used. However, research with orally administered clinical diets can be challenging since critically ill fish will often reduce or cease feeding, and there is therefore no swift and easy way to verify the outcomes of controlled study setups into field settings. The challenge thus remain to carry out and publish applied research under practical conditions to validate the findings done in controlled clinical trials, and thereby to quantify and/or predict the health effects of dietary interventions for everyday salmon farming.

Critical evaluation of research design and methods used in health feed trials

Different fields of science have their tradition of methods and approaches, including strategies for study design and statistical analyses of data. As described in the previous sections, many of the studies cited include various techniques for experimentally challenging fish with an infectious agent after or during various dietary interventions. As opposed to the situation in experimental animal medicine or in many terrestrial animal studies, feed administration must take place in groups, and cannot be measured or even estimated individually without exceptional efforts. While experimental infection may be performed on individual animals per inoculation, this route of exposure is highly artificial as several of the natural defence barriers of the fish (epidermis and mucous membranes) are surpassed. More realistic challenge conditions (via the water) often recommended for applied research can only be applied to groups of fish and not individually. Even individually exposed fish (e.g. by inoculation) will eventually interfere with each other via shedding of the infectious agent when released back to the same tank or pen. This situation implies specific challenges for trial design and statistical analyses of data, as the basic criteria of independence between units of concern are broken.

Evaluating various feed additive supplementation and other formulations used in control of infectious diseases therefore represents a special challenge for the experimental as well as for epidemiologically oriented scientist. There is a clear distinction between challenge trials under well-controlled experimental conditions and studies under true field conditions when infectious pressure, waves, the total aquatic microbiota and tidal currents contribute to a highly diverse disease pattern. If any dietary intervention aspires to demonstrate a valid strategy to control infectious diseases in the sea farming situation, the ultimate documentation needs to be collected from both controlled challenge trials and field studies under realistic and representative conditions. While design options are numerous for controlled trials, field trials mainly develop into an observational cohort study, possibly involving many pens and sites (multi-centre).

To quote a paper by Georgiadis, Gardner and Hedrick 2001[5]:

Fish pathologists often use experimental-challenge studies, in which the clinical, serologic and pathologic patterns of infectious disease are evaluated. Valuable data on the pathogenicity of the infectious agent as well as the susceptibility and response of the fish can be generated from such studies. Moreover, efficacy of

vaccines or therapeutic interventions can be assessed in laboratory clinical trials, where study fish can be challenged with the pathogens under defined and controlled conditions and the effects of extraneous variables are minimized. The presentation of an infectious disease is often quite different in the field than under laboratory conditions. Variable or prolonged exposure to the pathogen might be expected in natural outbreaks; stocking densities might also be greater and water quality poorer in an aquaculture setting than in the laboratory. Furthermore, other infectious (or non-infectious) diseases that might be present concurrently could influence the observed outcomes...

Therefore, it is imperative that results of laboratory studies are verified and assessed in field studies (either observational, or more often on-site clinical trials)...

These studies, again, need to be designed and conducted with the cooperation of the fish pathologist and the epidemiologist.

Reading the literature, there is sometimes an apparent lack of consideration for statistical design and analyses when concluding on data for the effect of nutritional intervention. Comparing groups of fish in one or two tanks with groups in another 1-2 tanks receiving differing feed formulations may be severely misleading, especially for infectious disease outcomes. Within-group infection patterns are often highly variable and thus few replicates will reduce the likelihood of detecting real and relevant differences. Of special concern in controlled studies as well as in field trials is that infectious diseases spread between individuals in a group, and hence the independence criterion is typically broken if individual fish is chosen for the statistical unit. For many infectious diseases, this means that the group in a pen or tank collapses into one single unit of concern for the statistical analysis. Furthermore, the route of challenge; injected, oral, immersion or by cohabitant exposure (infected fish being released into the tank to shed microorganisms) are of varying relevance for field conditions. A protective factor of importance under heavy infectious pressure in controlled trials may not be important in field conditions, or vice versa.

Another question often not understood properly is that employing a mixture of outcomes measured at individual level (death/symptoms) and outcome or exposure values obtained at group level (expressed as an average of group members or measured on the group as a whole) may lead to a problem called “the ecological fallacy” (also called inference fallacy). This occurs when associations measured on group or aggregated levels *per se* are believed to occur also on individual level, or vice versa. Examples of this situation may be linked to measuring important covariates as temperature, currents etc. as daily averages on pen or site level where the real story

is a highly fluctuating pattern. If a group of pens shows a reduced average growth, there may still be large variance differences between pens of more interest than the average measures. A main obstacle to harvesting data on same level is of course the sampling problem in field settings where a true random sample may be difficult to collect.

Another aspect of troublesome areas in field trials is that we may measure an effect as e.g. that pens with larger salmons will be found with more lice. This may not be anything else than a joint temperature effect on lice and growth, and we have found examples where this effect is not found on individual fish inside a pen. This effect is sometimes referred to as the contextual effect (Dohoo et al. 2008)[3].

The lack of independence between individuals, and the ecological fallacy problem are two critical points encountered in many of the papers we have assessed. The main statistical effect (if this is not accounted for) may be an artificially low p-level and hence a claim of “significance” where it may be a pure statistical artefact. Situations where contextual effects are encountered may lead to an opposite conclusion depending of whether this is accounted for or not.

Whatever design is used in laboratory or field experiments, basic criteria as control groups and realistic feeding and challenge conditions need to be followed. If the aim is to document a real effect of any dietary intervention there is no easy way. The extensive literature referred to in this report using specific feed formulations to control infectious diseases not only refers to small scale trials, but also to studies with a wide range of measured outcomes. Generally speaking there are two main approaches:

1. Using a “real” or **direct clinical outcome** by challenging the experimental fish with a pathogen and recording disease symptoms/ death/ pathology on individual or group level
2. Using **indirect outcome** measures as various immune- enzyme- or gene activation parameters, stress hormones, weight or growth rates as well as general mortality (in contrast to mortality in a disease challenge situation), economic performance etc. as proxy for measuring clinical outcomes

With the last approach, it is difficult, if not impossible, to make any predictions regarding a possible outcome in a disease situation. This will vary between types of pathogen and/or specific diseases. For example, measuring a large number of immune parameters following intervention

(or associating a large number of parameters with increased survival in a challenge trial) often leads to “fishing” for statistical effects among a large number of outcomes, sometimes with results that rarely can be expected to be reproduced clinically in the field. We want to stress that more reliable results from a controlled study can only be obtained by:

- developing and stating clearly the hypothesis behind the study
- working mainly on the direct outcome(s) – death, disease symptoms or establishment of generalised infection caused by specific infectious agents
- limiting the number of indirect outcomes measured to those that are highly relevant

As a side comment, it is important to acknowledge the need for defining relevant criteria on which to assess efficacy of a dietary treatment for a specific disease situation before starting a controlled or field study. The involvement of trained medical personnel (e.g a fish pathologist) in such studies is therefore strongly recommended to aid in this. Also, the effect of manipulating or supplementing a diet on individual fish can be relatively small in magnitude, and the results may only become relevant after summarizing the effect on a large number of individuals in the group, including interactive (“population-“) effects. This is typically in contrast with vaccine studies, where both individual and group effects should be of considerable magnitude. If trying to prove small outcome effects on group level, there is plenty of room for abuse of statistical methods leading to a claimed “significance” of biological or practical relevance.

Most studies dealing with animal feed rely upon traditional approaches for statistical analysis using simple parametric or non-parametric group comparisons or ANOVA-based methods with a pre-defined level of significance, being perceived a general “standard” in many scientific fields. The strict requirements underlying these methods, however, often remain non-implemented during study planning. As shown by Dohoo et al., 2008)[3], the p-value from ANOVA based approaches may be systematically biased in observational (epidemiological studies) and should be used with great caution.

Most studies of the use of feed for disease control are presented either by traditional nutritional scientists lacking education about infection dynamics, or by basic research scientists most interested in the function of (parts of) the fish immune system. Both these groups seem to heavily favour parallel-group designs, combined with rather old-fashioned statistical paradigms. Nutritional scientists have a long tradition in measuring growth performance, but their statistical methods may give strongly biased results if used on fish in a concurrent infectious disease

situation. Basic scientists rely predominantly on simplified experimental setups with as few variable factors as possible, and more sophisticated statistical techniques and study designs based upon multifactorial analysis of outcome measures are rarely utilized.

There is no way to escape the complexity of infectious diseases and as a minimum proper adjustment for covariates and confounders should be undertaken. Above all, we want to repeatedly emphasize the importance of always considering relevant group effects when studying infectious diseases. The main consequence of not doing so will be that results are reported as statistically significant not because of the factor under study, but because of unknown and uncontrolled population phenomena. A focus on exploratory analyses before the main statistical analyses may reveal this, but most important is to use proper techniques as multivariable regression with adjustment for cluster and group effects. Without doing so results can typically not be reproduced in another study, leading to confusion and undermining the trust of the users and society in the scientific community as a whole. Studies using multiple outcomes of indirect disease measures should focus more on using proper multivariate statistical techniques to identify response patterns than focusing on a series of single outcomes.

Are observational cohort studies the answer?

It is easy to acknowledge that in order to reliably estimate clinical effects in real-life practical aquaculture operations, data need to be collected under true field conditions. While routinely collected data may represent important sources of information, it seems clear that only a combination of results from routine measurements, combined with properly designed specific studies may exploit the real strength of using tools from the epidemiological toolbox.

A recent example also of interest for feed trials is the evaluation of the PD-free program⁵. In the critical situation experienced the last years, the industry has continuously tried to improve health management, introduced various feed intervention strategies, and then using also a vaccine with limited documentation of protective effects. The investigational strategy chosen in the mentioned report has been to obtain data from a subset of fish farms in the endemic zone,

⁵ http://www.vetinst.no/content/download/8301/99934/file/Rapportserie_11-14_Vurdering_av_effekter_av_PDfri_prosjektet_2008-2010.pdf

including PD outbreaks, the occurrence of other diseases, and a range of environmental and economic parameters, with overall cumulative mortality being the most important. The report concludes that the vaccine moderately reduces the risk of a PD outbreak, but more interestingly, also in reducing the overall mortality. Working with a range of databases where mortality and its causes has been in focus, Aunsmo et al. 2008[10] showed, however, that overall mortality may be a severely biased parameter. If we expect that operational management interventions alone would reduce the risk of PD, any intervention performed concurrently would be expected to show an effect. Clearly the results cannot provide a clear documentation of vaccine effect, as the study violates most criteria that are needed to substantiate a claim of causality. This has to be emphasised, because any intervention such as vaccine or health feed should definitely be demonstrated to be causal and not a spurious statistical association for improved health. The report cited above is used as support for use of the PD vaccine in the endemic zone. Data were not collected at sufficiently detailed level to include dietary intervention in the analysis, but due to methodological weaknesses pointed out above, we hypothesise that most likely the same would have been found – that functional diets are associated with reduced PD infection and mortality. We therefore advocate field studies to evaluate dietary intervention should primarily be performed using a controlled cohort design where the intervention in question is compared at each site over a relatively large number of sites with other factors kept as stable as possible in the study units. Surprisingly enough, studies have not been carried out for PD or other diseases in today's industrial salmon culture – in spite of the claimed effect of feed, vaccine or management in limiting or controlling the disease.

Suggestions for design and statistical methods to be used

For in-depth literature on this topic, we refer to the textbook by Thrusfield (2007; pp. 289-304) where various aspects of controlled and field trials including meta-analysis are covered. Also the textbook by Dohoo et al. (2008[3] provides excellent guidance for planning of studies and especially statistical analysis of observational data.

Types of studies and their use

We approach this discussion by distinguishing between the experiment and various trials:

1. the small-scale **experiment** where general aspects of immunology, gene transcription, disease patterns etc. are investigated under optimally controlled conditions, with or without experimental infection;
2. the **controlled trial**, where one or more specific treatments are to be tested under conditions that are as close as possible to those relevant in the field with an appropriate experimental exposure to infection, often on a smaller scale using isolated fish groups
3. **the field trial or cohort study** where groups in relevant rearing units are observed under natural conditions under planned intervention but with only natural disease challenge

Both controlled trials and field trials may be used to study disease preventive effects, while more indirect disease modifying effects of interventions that may have relevance for the clinical outcome (blood parameters, immune responses, gene expression levels etc.) are typically studied during experiments and controlled trials. In our opinion, experimental studies are more likely to produce spurious associations in particular when researchers study a wide range of outcomes and/or interventions. Generally speaking, experiments on disease prevention and disease modification always need to be repeated and the outcomes verified in both controlled and field trials. We therefore suggest these as the logical steps in an investigation series to document feed effects on disease:

1. A pilot experiment may be necessary to assess relevant outcomes that should be measured and to establish the number of fish and replicates needed for adequate power in subsequent trials
2. Solid support for relevant disease-specific prevention or modification effects in combination with the registration of relevant indirect outcomes (blood parameters, immune responses, gene expression levels etc.) in a controlled trial setup
3. Final documentation and quantification of clinical effects from large scale field trials / cohort studies

Only after all these steps have been conducted, may claims of health promoting effects be considered well-justified.

There are several groups of design options for field or cohort studies including parallel group-, cross-over-, sequential-, and factorial designs. For our purpose in studying dietary intervention on infectious disease, we will focus on two options – the parallel group (standard) trial and the cross-over trial. In a field trial, we generally name the parallel group design a “cohort design”. The other designs may be appropriate in the experimental stages of an investigation. Some problems and risks are inherent for any field trial, such as the absence of a disease outbreak, multiple infections occurring simultaneously, and inappetence of fish during disease outbreak. Regarding the latter, it is impossible to precisely control or record individual feed intake of fish held in groups (maybe except when using highly specialised and small-scale methods). However, marking options such as individual external or internal tags or other marking techniques of identifying individuals and registering the growth performance of individual fish during the course of the trial can serve as an indirect measure of feed intake and justify whether a distinct intervention has had an effect.

Design of controlled trials

Outcome analysis from controlled trials should always be done using a statistical model where potential group random effects are adjusted for. This typically involves the tank, pen or whatever group of fish are physically together during the trial. We recommend using a mixed effect regression model, whatever outcomes that will be measured. This allows a statistically valid interpretation and avoids false “significant” associations to be claimed. Careful planning and sample size calculation should be undertaken, preferable by estimating the random effect from pilot experiments or literature review.

Parallel design

The simplest approach to a controlled trial will be to test the trial feed vs. a control feed by running a randomized allocation of equal number of groups for each group. Figure 1 shows this in a simple 3+3+3 design with random allocation of treatment to 9 groups. Three groups in each treatment represent the absolute minimum for statistical analyses, and the described design will have a limited power in detecting feed effects. If individual data are recorded, a proper sample

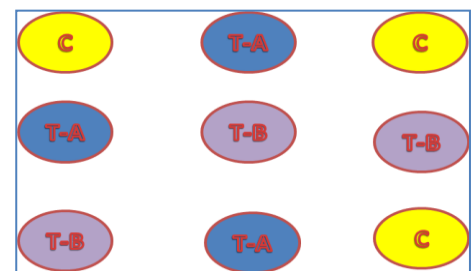


Figure 1. Simple parallel design with two trial feeds (T-A, T-B) and one control (C)

size per replicate groups must be used and the group effect must be adjusted for using random effect statistical models (Dohoo et al. 2008)[3]. If one measure per group is used, several groups are needed, and a proper sample size calculation should be undertaken before the study. Generally speaking, for infectious diseases, the best is to study multiple groups and reduce the size of each group. The parallel design may be used both for measuring prevention effect and disease modification effect.

Cross-over design

The cross-over design may be appropriate if the aim is to measure the prevention effect and disease modification/treatment effect in the same experiment, or a combined preventive and alleviating effect. This design encompasses changing the treatment between before and after challenge/disease occurrence. A simple setup with four trial groups + the control group is shown in Figure 2. Notice that we recommend using a standard feed for comparison (reference group) no matter which design is used. If only one experimental feed is to be evaluated the design will be simplified, but only by one group in total down to four. The

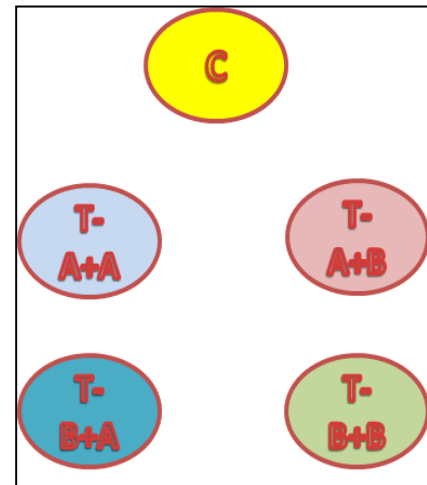


Figure 2. Design groups in a cross-over design with one control feed C and two trial feeds T-A and T-B

need for replicates are the same, and there is a special need for evaluating sample size before the

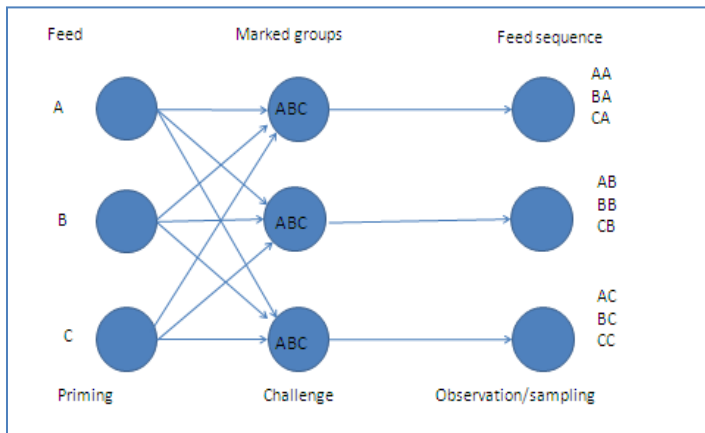


Figure 3. Proposed cross-over design of controlled trial with two test and one control feed, and group marking before challenge.

trial, as many additional parameters need to be estimated (separate effects + interactions). Major limitations to this approach are the previously mentioned inappetence in a disease setting.

An even more comprehensive cross-over design for controlled health feed trials (with group marking) is shown in Figure 3.

Design of field trials

The principles behind a field trial would be much the same as for a controlled trial, but options are more limited, and the only practical approach would be to run a parallel design as a cohort study. In selecting pens at a site for inclusion as treatment or control pens, any bias between the units allocated to test or control treatment should be avoided. Random allocation among matching pens (same origin and size of fish, same vaccine, same pen size etc.) is mostly required in order to achieve relatively unbiased conditions, and a trial setup is shown in Figure 4.

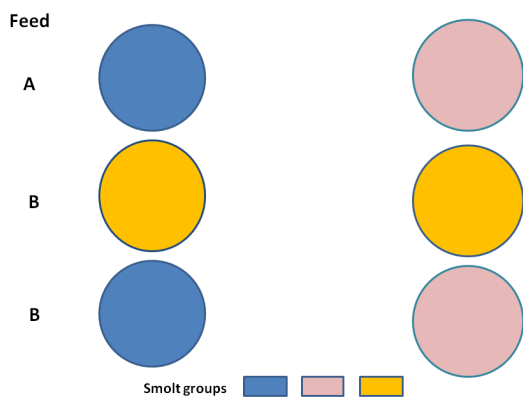


Figure 4. Field study setup with six pens and two feeds, with matching on smolt origin. Feed allocation randomized within smolts from hatchery 1 (blue) and from hatchery 2 (pink), respectively, while further deliveries (yellow) were allocated to feed group by convenience.

Field trials can be extended to encompass multiple sites within larger geographical areas, where a large or small fraction of the pens at each site are treated. The main point is to make sure there is a sufficient number of pens for each group of interest, taking the anticipated level of effect into account. Subtle effects anticipated on disease outbreaks, as is the case for at least some nutritional interventions, would need a larger set of pens/ sites than e.g. a field trial on larger (e.g. vaccine) effects. A problem affecting all marine aquaculture production is that effects may be season/ temperature/ size/ life stage related, and thus field trials should be planned tanking these specificities into account to reduce the number of pens and sites to a manageable figure.

Statistical techniques

Many of the experimental studies cited may have clear and identifiable hypotheses, where a traditional hypothesis driven conclusion (observed effect being statistically significant or not) may be appropriate. However, going into the field with a controlled or field study setup, we are first and foremost concerned about the magnitude of a potential effect, and less about their statistical significance. Moreover, the sample size calculated as required for hypotheses testing may be difficult to perform in the field study setting. The simple answer to this situation is that we strongly recommend various multivariable regression techniques to be used in analysing data from controlled or field trials. This estimated effect can be reduced cumulative mortality, lower probability of clinical disease outbreak, or longer time to death or disease outbreak. For more information about these techniques we refer to the textbook by Dohoo et al. (2008)[3]. Modern regression techniques allow any outcome (numeric, binomial, ordinal, nominal or time to event) to be modelled, and allows for adjustment of random effects and pen effects using hierarchical (multilevel) regression techniques. Re-analysing data using these techniques may give very different results than simpler analyses often used in the cited papers. Ideally, we would recommend that some of the data from controlled feed trials should be re-analysed using these statistical techniques. Papers presenting multivariate data (several outcomes) represent a special challenge, but also here a range of techniques allow us to make more sound statistical inferences than previously possible. This may especially be a concern for experimental studies, and a way to avoid going into expensive controlled trials without a substantial claim of effect.

Meta-analysis

A standard approach to deal with uncertainties is to establish a meta-analysis platform (Dohoo et al. 2008)[3] where results from different studies can be compiled and compared. This is typically done in human clinical studies and claimed to be some kind of Gold standard for clinical trials, and a specialised system – the Cochrane Collaboration – is set up to perform these kind of studies.

An especially interesting aspect of the meta-analysis technique is the ability to detect publication bias and under-reported results. We therefore feel that in principle, any claim of a substantial and

predictable effect of intervention linked to feed regimes on disease should be supported by a systematic compilation of results from various studies, also if needed with different designs.

Further comments on design

Our recommendations are largely based upon being more stringent in passing from the experimental setting to a controlled or field trial setting, and changing the paradigm of design and statistical analyses when doing so. Moreover – one should not place total trust in results from one single experiment or trial without comparing it with other studies, or repeating the study.

Possibilities

The increased possibilities opened through our suggested approach are that different studies (experimental, controlled and field trials) will show various aspects of results, and thus jointly constitute a firmer basis for justifying a causal claim for any specific feed intervention. A strict approach to design and statistical analyses will not solve all problems, but will increase the likelihood of revealing the true magnitude of differences between feed formulae, which is essential in order to cost optimize industrial aquaculture production. There are more than sufficient companies, sites and pens in Norway to be able to run trials of statistically adequate size if the industry establishes proper collaboration structures inside and between companies. Several trial farms and sites exist and should be used more extensively for controlled trials, while normally managed sites should be used for the larger scale, less controlled studies.

Limitations

The major limitation in experimental studies and controlled trials using challenge techniques is the lack of ability to predict the magnitude of effect under field conditions. We therefore strongly recommend that all interventions linked to feed should be finally evaluated using a large scale parallel (cohort) design. The effect of functional feeds may not be large or robust enough to overrule disturbances such as fluctuating water temperature and/or O₂-saturation, current, other infections etc. which are not present under controlled experimental conditions. This may result in differing outcomes obtained between controlled trials and field trials of limited size.

Field trials show obvious limitations due to the unpredictable conditions, in particular whether or not an infectious disease challenge will occur. At low incidence of disease, a parallel

group/cohort design must be very large to be able to provide enough information from infected sites or pens. Alternative designs such as case-cohort or similar may help here, but are often difficult to carry out in practice. Another limitation of field studies is that site specific data may not have sufficient sensitivity for detecting economically important effects in the population, as even a minor growth enhancement may be important due to the enormous biomass.

List of attachments

Attachment 1: Reference list

Attachment 2: Anne Marie Bakke: Alleviation of feed-induced enteric pathologies