Replacement of fish protein in fish feed

- Effects on the yeast flora in the gut of Arctic charr (*Salvelinus alpinus)*

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Swedish University of Agricultural Sciences, SLU
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Ersättning av fiskprotein i fiskfoder – Effekter på jästfloran i tarmen hos röding (Salvelinus alpinus)

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Abstract

The aim with this study was to investigate how feeds with different protein sources influence the levels and composition of the yeast flora in the gut of Arctic charr (*Salvelinus alpinus*). Arctic charr were fed for two weeks on a control diet (C) with fishmeal (FM) or experimental diets with 40% FM replaced by yeast (*Saccharomyces cerevisiae*) (Y), meal from blue mussel (*Mytilus edulis*) (M) or a feed with Baltic Sea originated protein; 33% FM, 33% mussel meal (MM) and 33% *S. cerevisiae* referred to as Baltic blend (BB). The effect of the different feeds was evaluated based on fish growth and the effects on the microbial population, with a focus on yeasts, in different parts of the gut (stomach (S), pylorus (P), mid intestine (MI) and distal intestine (DI)). Differences in the development of the yeast flora composition and yeast loads were investigated using agar plate counts and yeast species identification using polymerase chain reaction (PCR) amplification combined with sequencing of the 28S rRNA gene. The amount of bacteria and moulds were also estimated when examining the agar plates. In addition, the different feeds were analysed for yeast load, yeast species composition and diversity. The study showed that there were differences in yeast load in the gut linked to diet, where the diet containing *S. cerevisiae* (Y) had a higher yeast load compared to the other diets both before (C: $p = 0.003$, M: $p = 0.016$, BB: $p = 0.0007$) and after the diet intervention (C: $p = 0.024$, M: $p = 0.001$, BB: $p = 0.001$). Differences in amount of yeast could also be linked to time (i.e., before and after) ($p = 0.0009$) with greater yeast loads at the end of the experiment, where the yeast load in the gut ranged between 4.1–7.5 log CFU g^{-1} after two weeks of dietary treatment. However, no differences between gut segments were found on yeast load or yeast composition. The domination yeast species found in gut in all diets were *Debaryomyces hansenii* (68–70% of yeast isolates) followed by *Debaryomyces sp.* (19–24%). Both *D. hansenii* and *Debaryomyces sp.* were more abundant at the end of the study. No differences in yeast composition were found between diets. In all feeds, *D. hansenii* was found and *S. cerevisiae* was detected in all feeds except in the control feed (C). The feed with 40% FM replaced by *S. cerevisiae* (Y) had the highest yeast load with *D. hansenii* as the dominating yeast specie. The different experimental diets did not impact weight or length between the fish in the study after two weeks trial. The study design lacked tank as a factor for statistical analysis. Hence, the results might only be viewed as indications. Further research is necessary for continued understanding of the feed impact on the yeast flora in gut of Arctic charr.

Keywords: microbiota, *Saccharomyces cerevisiae*, fishmeal, mussel meal, *Mytilus edulis*, aquaculture

Sammanfattning

Syftet med studien var att undersöka hur foder med olika proteinkällor påverkar mängd och sammansättning av jäst i tarmen hos röding (*Salvelinus alpinus*). Röding utfodrades under två veckor med ett kontrollfoder (C) innehållande fiskmjöl (FM) eller tre försöksdieter där 40% av FM byts ut mot jäst (*Saccharomyces cerevisiae*) (Y), musselmjöl (MM) från blåmussla (*Mytilus edulis*) (M) eller Baltic blend (BB), vilken består av en mix av proteinkällor; 33% FM, 33% MM och 33% *S. cerevisiae*. Effekten av de olika fodren utvärderades genom att mäta fiskarnas tillväxt och påverkan på den mikrobiella populationen i olika delar av tarmen (magsäck (S), pylorus (P), mellantarm (MI) och distaltarm (DI)) med fokus på jäst. Skillnader mellan påverkan på jästfloran med avseende på jästmängd och sammansättning undersöktes via räkning av kolonier på agarplattor samt med polymeras kedjereaktion (PCR) och sekvensering av genen 28S rRNA som detekterats och amplifierats från kolonier på agarplattorna. Mängden bakterier och mögel skattades vid utvärderingen av agarplattorna. De olika fodren analyserades med avseende på jästmängd samt jästartsammansättning och mångfald. Studien visade att det fanns skillnader mellan jästmängd i tarmen kopplat till fodertyp, där gruppen som utfodrats med *S. cerevisiae* (Y) hade den största jästmängden jämfört med de andra grupperna både före (C: $p = 0.003$, M: $p = 0.016$, BB: $p = 0.0007$) och efter introduktion av försöksdieterna (C: $p = 0.024$, M: $p = 0.001$, BB: $p = 0.001$). Skillnader i jästmängd kunde också kopplas till tid (dvs. före och efter) ($p = 0.0009$) med högre jästmängd vid försökets slut där jästmängden i tarmen varierade mellan 4.1–7.5 log CFU g^{-1} efter två veckors utfodring med olika fodren. Inga skillnader hittades dock mellan de olika tarmdelarna med avseende på jästmängd eller jästsammansättning. Den dominerade jästarten som hittades i tarmen oberoende av fodertyp var *Debaryomyces hansenii* (68–70% av jästisolaten) följt av *Debaryomyces sp.* (19–24%). Förekomsten av både *D. hansenii* och *Debaryomyces sp.* var rikligare vid studiens slut. Inga skillnader i jästsammansättning kunde påvisas mellan de olika fodertyperna. *Debaryomyces hansenii* hittades i alla foder och *S. cerevisiae* fanns i alla foder utom kontrollfodret. Fodret med 40% FM utbytt mot *S. cerevisiae* (Y) innehöll den största jästmängden med *D. hansenii* som den dominerande jästarten. Studiens utformning saknade tank som en faktor vid statistisk analys och därför bör resultaten endast ses som indikationer. Vidare forskning är nödvändig för ytterligare förståelse av fodrets påverkan på jästfloran i tarmen hos röding.

Keywords: mikrobiota, *Saccharomyces cerevisiae*, fiskmjöl, musselmjöl, *Mytilus edulis*, akvakultur

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Abbreviations

AA	Amino acids
ADC	Apparent digestibility coefficients
BB	Baltic blend
CFU	Colony forming units
CP	Crude protein
DI	Distal intestine
DM	Dry matter
FAO	World Food and Agricultural Organization
FM	Fishmeal
FO	Fish oil
GI	Gastrointestinal
LAB	Lactic acid bacteria
MI	Mid intestine
MM	Mussel meal
NCBI	National Center for Biotechnology Information
PAST	Paleontological Statistics
PCA	Principal component analysis
PC	Principal component
PCR	Polymerase chain reaction
YPD	Yeast peptone dextrose

1. Introduction

As the world population grows, challenges such as poverty, hunger and malnutrition expand (FAO 2022). More than 800 million people around the world suffer from hunger today. In order to face these challenges, the urge for nutritious food has become more important. Being recognized as an important contributor to the world food system, aquaculture and wild fisheries provides the world population with essential fatty acids and animal protein, playing an important role in fighting hunger around the globe (FAO 2022).

Aquaculture is a rapid growing industry with an average annual growth rate of 6.7 percent over the last 30 years (FAO 2022). Between 1990 and 2020, the annual output of the aquaculture sector expanded with over 600 percent (FAO 2022). Thus, the feed input and supply of nutrients for fish must meet the demand at a similar rate (Tacon & Metian 2008; FAO 2011). By 2050, aquaculture industry is predicted to expand to almost double its current production volumes (FAO 2022). To make such an expansion possible, large quantities of suitable feed ingredients that could provide all essential amino acids, vitamins, minerals and omega-3 fatty acids, needs to be available and affordable for the industry (FAO 2022).

Traditionally, fishmeal (FM) based on marine ingredients has been the main protein source in diets for farmed fish (Tacon & Metian 2008). However, availability of both FM and fish oil (FO) have declined over time, resulting in increased prices (FAO 2012). Concerns regarding the sustainability to use FM and FO as feed ingredients is also a discussed subject (FAO 2011). In 1995, the World Food and Agricultural Organization (FAO) agreed on the Code of Conduct for Responsible Fisheries, aiming towards increasing sustainability for wild fisheries (FAO 1995). However, the grade of implementation of the FAO Code varies among countries (Pitcher et al. 2009). Competition of feed ingredients with livestock and humans is also discussed within sustainability of fish feed (FAO 2011). The production of FM and FO processed out of by-products from fisheries and aquaculture has been progressing with the purpose to replace wild catch (FAO 2022). However, aquaculture needs to make it less dependent of marine biomass for feeding of farmed fish (FAO 2022). To ensure sustainability within aquaculture production, alternative protein sources to FM should be further reviewed (FAO 2011).

Yeast and other microbial-derived feed ingredients are potential substitutes for FM in fish feed (FAO 2012). The yeast *Saccharomyces cerevisiae* has shown promising results as protein replacement for salmonid fish (Barnes & Durben 2010; Vidakovic et al. 2016; Hines et al. 2021). The protein-rich blue mussel (*Mytilus edulis*) has also been an interesting alternative to replace FM for many years (Berge & Austreng 1989; Kiessling 2009). The Aquabest project (2007–2013), partly financed by the European Union, focused on developing aquaculture in the Baltic Sea region in a responsible way. As a part of the Aquabest project, FM and mussel meal (MM) from the Baltic Sea region, fed to Arctic charr was evaluated (Carlberg et al. 2014). Apart from the Baltic Sea originated feed ingredients, the feed also contained *S. cerevisiae* as a protein source. This feed compound was called Baltic blend (BB). To enhance sustainability of the feed, the protein rich feed ingredients used for replacing FM should be unattractive as human food products (Carlberg et al. 2018).

The microbiota is the complex microbial community that inhabit the surfaces and cavities of the body that are open to the surrounding environment (Romero et al. 2014). The gastrointestinal tract holds a large proportion of the microbiota, influencing biological processes of the host. The fish gut microbiota can be affected depending on diet (Hoseinifar et al. 2011; Nyman et al. 2017). Studies have shown that dietary yeast can colonize fish gut and affect both diversity and conformation of gut yeast and bacteria (Waché et al. 2006; Hoseinifar et al. 2011; Huyben et al. 2017[a]; Nyman et al. 2017). The previous studies have mainly been performed on rainbow trout. The impact on the microbiota when fed various feed components is rather unexplored for Arctic charr (Nyman et al. 2017).

1.1 Aim

The aim with this study was to investigate how feeds from different protein sources (FM, *S. cerevisiae*, MM, or the feed compound BB) influence the levels and composition of the yeast flora in different parts of the gut of Arctic charr. The main focal point was on live yeast culture in faeces. Growth performance of the fish was also investigated.

1.1.1 Hypothesis

The hypothesis was that the yeast flora in the gut would differ between the different treatments, based on the fact that some of the feed included *S. cerevisiae*. This assumption included both strains of yeast present in the gut and number of colony forming units (CFU). Presumably, the fish fed with *S. cerevisiae* would have a higher number of present yeast cultures in faeces compared with the other treatments.

2. Background

2.1 Aquaculture

In 2020, the global aquaculture of aquatic animals was estimated to 88 million tonnes (FAO 2022). It corresponds to 49% of the total production of aquatic animals in the world (178 million tonnes). Asia, being the dominating continent in aquaculture for decades, stood for more than 90% of the total aquaculture production in 2020. Aquaculture continues to grow and has over the last two years grown faster than capture fisheries. Finfish farming accounts for the largest part of aquaculture (FAO 2022).

Approximately 55 million tonnes of the total global production of aquaculture in 2020 were produced in inland waters (freshwater aquaculture) (FAO 2022). The prerequisite of inland aquaculture varies around the world, using different type of facilities and methods for culture. Both technologies, management systems, operations and integration with other farm capabilities varies depending on country. On a global point of view, the most common farm system used is earth ponds on land (FAO 2022). Fish farming systems in Sweden is most commonly open systems using net-pens in lakes or close to the shore (Jordbruksverket 2020).

Feed in intensive aquaculture production consist to the largest part of feed made by extrusion (SOU 2009:26; Huyben et al. 2017[a]). Extrusion is a feed manufacturing process, where the feed is subjected to high temperatures and pressure (Vidakovic et al. 2016). Extruded feed has environmental advantages compared to pelleted food, as the leakage of nutrients decreases as particle dissolution in water slows down (SOU 2009:26).

2.2 Arctic charr (*Salvelinus alpinus*)

Arctic charr is a salmonid fish that have been cultivated in Sweden since the 1980's, following the introduction of the Arctic charr breeding programme (Nilsson et al. 2010; Brännäs et al. 2011). Arctic charr is a cold-water species and cultivation facilities has mainly been located in the north of Sweden (SOU 2009:26). The water temperature required for farming of Arctic charr is below 15℃ for a good

cultivation climate. In Sweden, farming of Arctic charr is mainly based in lakes or water reservoirs (SOU 2009:26). In 2020, the production of Arctic charr in Sweden was approximately 1100 tons, making up for 11% of the total tonnage of farmed fish in Sweden (Jordbruksverket 2020).

When determining production costs, the stocking densities of fish are an important factor (Wallace et al. 1988). If stocked at high densities, the production cost per fish will decrease. However, other aspects such as sustained levels of mortality and growth must also be taken into account. Optimal stocking densities in production can be affected by age, size, feeding rate and water temperature (Wallace et al. 1988). Growth rates of Arctic charr can be affected by factors such as water temperature (Larsson & Berglund 1998) and stocking density (Wallace et al. 1988; Jorgensen et al. 1993). High growth rates have been shown at water temperatures ranging between 13 to 18°C, with a possible maximum at 15°C (Larsson & Berglund 1998). For Arctic charr, the optimal fish density has been shown to be higher than for other salmonid fish (Wallace et al. 1988; Jorgensen et al. 1993). However, the recommended stocking density is also depending on life stage of the Arctic charr (Wallace et al. 1988). It's been reported that Arctic charr fingerlings has maintained growth performance up to stocking densities of 100 kg m^{-3} (Wallace et al. 1988; Jorgensen et al. 1993). Jorgensen et al. (1993) showed that low stocking densities (<20 kg m^{-3}) resulted in depressed growth performance in Arctic charr. Growth rates in Arctic charr may also decrease with shallow water depths (30-40 cm) (Jobling et al. 1998).

An important aspect in order to reach optimal production results is the size of feed particles (Tabachek 1988). Factors such as acceptance, feed efficiency and growth can be affected by the feed particle size. Tabachek (1988) showed that weight increase, specific growth rate and feed efficiency of Arctic charr was significantly affected by feed particle size. In addition, the number of feed particles fed is of importance (Tabachek 1988).

2.3 Alternative protein sources in fish feed

For the individual fish farmer, the largest production cost is feed (SUO 2009:26; Kiessling 2009). There is a large interest of increasing feed efficiency within aquaculture, both for economic and environmental reasons. Also, the availability of traditional feed components such as FM and FO display a decreasing trend (FAO 2012). The availability of FM an FO is by a large extent dependent on the events of El Niño in the eastern Pacific (Hardy 2010). El Niño is a climate phenomenon, influencing the catch of wild fish (Hardy 2010; FAO 2022). In an El Niño year, FM production can decrease with more than 1000 000 metric tonnes (Hardy 2010). In 2020, 16 million tonnes of the global production of aquatic animals were designated for production of FM and FO (FAO 2022). However, the inclusion rate in feed for

farmed fish has resulted in a downtrend as a result of high prices and supply variations combined with pressure from the feed industry. In grower diets for salmons, FM constitutes less than 10% of the feed composition (FAO 2022).

Both plant protein and other protein sources such as microbes have been of interest as substitute for fish protein in feed (Kiessling 2009). Plant protein such as oilseed meals, grains and legumes, is commonly used in aquaculture as a source of protein in fish feed for salmonid fish (NRC 2011; Smith et al. 2018). The shift from FM and FO to plant ingredients has been necessary to meet the demand of the growing salmon farming industry (Ytrestøyl et al. 2015). Soy protein concentrate has been used as the main replacement of FM in feed for farmed salmon (Ytrestøyl et al. 2015). Soybeans has a good amino acid (AA) profile, suitable for feeding of fish (Chikwati et al. 2012), however a problem with soybean and products thereof, is the containment of antinutritional factors (Hajra et al. 2013). Also, sustainability must also be considered when replacing FM and FO with plant ingredients as most of these options can be consumed by humans (Ytrestøyl et al. 2015). The most sustainable alternative would be to use protein not suitable for human consumption in order to avoid competition between humans and animals for the same protein sources (Kiessling 2009). An approach to replace FM and FO in fish feed is to search for options lower in the ocean food chain (Kiessling 2009). Microorganisms, such as yeast, are not suitable for direct human consumption (Carlberg et al. 2014). Yeast, such as *S. cerevisiae*, has a suitable AA profile for fish, resembling to FM (Agboola et al. 2021). Studies has shown that FM has been successfully replaced with yeast up to 40% without reducing growth performance in rainbow trout (*Oncorhynchus mykiss*) (Hauptman et al. 2014; Huyben et al. 2017[a]) and in Arctic charr (Vidakovic et al. 2016). Blue mussels have also showed positive results when substituting 40% FM in feed to Arctic charr (Vidakovic et al. 2016).

2.3.1 Baker's yeast (*Saccharomyces cerevisiae*)

Saccharomyces cerevisiae also known as baker's yeast, is a food grade yeast used in various food production systems such as beverages and fermentative foods (Belda et al. 2019). *Saccharomyces cerevisiae* is a unicellular yeast, reproducing by budding (Stewart 2014). The species is extensively used in research and industry, being the most studied species of the yeast domain (Stewart 2014).

Multiple studies have been performed on *S. cerevisiae* as a protein source in aquatic feed for salmonid fish. Both intact and extracted yeast has been tested for feeding of fish (Rumsey et al. 1991; Øverland et al. 2013; Langeland et al. 2016; Vidakovic et al. 2016). The results of these studies have somewhat diverged. One study showed that moderate levels (40% of the crude protein (CP) in FM) of intact *S. cerevisiae* feed to Atlantic salmon (*Salmo salar*) reduced both nutrient utilization and growth performance (Øverland et al. 2013). Rumsey et al. (1991) showed that the nutritional value for salmonid fish increased by disruption of the brewer's dried

yeast (*S. cerevisiae*) cell wall. Langeland et al. (2016) also presented results indicating that a disrupted cell wall of *S. cerevisiae* had positive effects on digestibility in Arctic charr. However, Vidakovic et al. (2016) found that intact *S. cerevisiae* showed promising results as a possibility to replace FM in fish feed in Arctic charr, contradicting earlier findings. Though, partial destruction of the cell wall might have been caused by extrusion at the feed manufacturing, resulting in higher digestibility (Vidakovic et al. 2016). Supplementation with a fully fermented yeast culture containing *S. cerevisiae* resulted in reduced mortality in rainbow trout according to Barnes and Durben (2010).

2.3.2 Mussel meal

The blue mussel is a mollusc with function as a plankton filterer, which has been of interest as a protein source for farmed fish for more than 30 years (Berge & Austreng 1989; Kiessling 2009). The method used for farming blue mussels is by ropes placed in the ocean (SOU 2009:26). Free-floating mussel larvae attach to the ropes during reproductive season and grow into mussels. The period from larvae to harvest of full-grown mussels is around 18 months (SOU 2009:26).

The blue mussel has an AA profile suitable for substituting FM in fish feed and a high protein content (Berge & Austreng 1989). Using waste products from mussel farming e.g., mussels too small for human consumption, could be a potential approach for fish feed (Kiessling 2009). The fraction of mussels below marketing size will be sorted out during harvest and could represent up to 30-50% of the total harvest (Berge & Austreng 1989). In 2020, the mussel production in Sweden was approximately 2300 tons (Jordbruksverket 2020).

Usage of de-shelled blue mussels might enable higher levels of inclusion in diet (Langeland et al. 2016). Berge and Austreng (1989) found that the dry matter (DM) digestibility declined in rainbow trout with increasing inclusion quantities of blue mussels, which might be a result of high shell content. The shell contains approximately 80% ash, which mostly will pass through the gastrointestinal (GI) tract undigested (Berge & Austreng 1989). In the study by Langeland et al. (2016) on Arctic charr, inclusion of de-shelled blue mussels had a positive effect on the apparent digestibility coefficients (ADC) of DM compared to the reference diet with FM and soy protein. Growth performance in Arctic charr was not negatively affected when fed de-shelled blue mussels, which replaced 40% of FM in diet (Vidakovic et al. 2016).

2.3.3 Baltic blend

Baltic blend is a feed that contains 33% Baltic Sea-originated FM derived from sprat (*Sprattus sprattus*) and herring (*Clupea harengus*) (Carlberg et al. 2014). The feed compound also contains 33% *S. cerevisiae* and 33% Baltic Sea MM as protein

sources. Mussels from the Baltic Sea are dwarfed by the low salinity and do not grow as fast or as large as North Sea mussels (Tedengren & Kautsky 1986). Usage of mussels below marketing size, have been mentioned as an alternative protein source in fish feed (Kiessling 2009).

Carlberg et al. (2018) evaluated growth in Arctic charr fed BB during a full production cycle combined with a sensory test for consumption. Fish fed BB had a growth reduction of 11.5% compared to the reference diet and lower feed digestibility (Carlberg et al. 2018). Intact *S. cerevisiae,* included in BB, has been shown to affect digestibility negatively (Rumsey et al. 1991; Carlberg et al. 2018).

2.4 Gastrointestinal microbiota in fish

The GI tract consists of a microbial community of yeast, bacteria, archaea, viruses and protozoans (Romero et al. 2014). The normal microbiota of the gut is often described as the collection of microorganisms that inhabit the GI tract under normal circumstances (Berg 1996). The microbiota of the GI tract influences various biological processes of the host, such as feed digestion, nutritional functions, immunity, disease resistance and gut development and persistent health of the organ itself (Berg 1996; Romero et al. 2014). Studies on microbiota in fish gut has mainly focused on bacteria while there is more limited data on yeast in the GI tract (Romero et al. 2014). Identified as a part of the normal microbiota of fish, yeast can vary in both species' composition and amounts (Gatesoupe 2007). The natural quantities of yeast in fish gut can vary from non-detectable levels up to 10^7 CFU g^{-1} (Gatesoupe 2007). It has been shown that the GI tract of rainbow trout can hold dense populations of yeast (Andlid et al. 1995).

Yeast found in the microbiota in fish gut can be classified into two phyla of fungi: Ascomycota and Basidiomycota (Gatesoupe 2007). In rainbow trout, the Ascomycota yeasts; *Debaryomyces hansenii*, *S. cerevisiae*, *Candida* spp. and the Basidiomycota yeast *Leucosporidium* sp. have been found dominating the natural yeast flora in gut (Gatesoupe 2007). Andlid et al. (1995) also found *Rhodotorula rubra* and *Rhodotorula glutinis* when isolating yeast from farmed rainbow trout. Huyben et al. (2017[a]) identified new species in the GI tract of rainbow trout; *Candida zeylanoides*, *Cryptococcus carnescens*, *Rhodosporidium babjevae* and *Rhodotorula graminis*. The precision of gene sequencing of yeast has resulted in new species being identified and as the methodology evolves, it likely to identify even more yeast species in fish gut (Huyben et al. 2017[a]).

2.4.1 Dietary effect on microbiota in fish gut

The impact of diet on the microbiota in the GI tract of fish has been investigated by numerous studies, mainly focusing on bacteria (reviewed by Romero et al. 2014).

According to Waché et al. (2006), certain yeast introduced with feed can colonize the GI tract of fish. It has been shown that yeast can colonize the intestinal mucosa of fish (Andlid et al. 1995). However, it is not evident that yeast introduced as feed will be able to colonize in the fish gut (Gatesoupe 2007). Hoseinifar et al. (2011) reported that the microbiota in fish gut might be affected by inclusion of inactive *S. cerevisiae* in feed, as fish fed yeast indicated increased levels of lactic acid bacteria (LAB) in the GI tract. Lactic acid bacteria (LAB) are normally considered as beneficial members of the microbiota with the ability to suppress and antagonize fish pathogens (Ringø & Gatesoupe 1998; Ringø et al. 2005; Balcázar et al. 2007; Hoseinifar et al. 2011).

Changes in the microbiota between different gut sections was investigated by Nyman et al. (2017), when Arctic charr was fed MM, *S. cerevisiae* or fungi (*Rhizopus oryzae*) as feed components. As the gut sections have diverse physiological functions and enzymes present, the substrate for microorganisms would be different (Nyman et al. 2017). There was no evidence that the microbiota differed in composition or diversity between gut sections. However, the microbiota diversity in gut was higher in fish fed microbe diets compared to a FM diet (Nyman et al. 2017). Nyman et al. (2017) also found that the microbiota composition in fish gut differed when fed MM compared to when fed *R. oryzae* or a *S. cerevisiae* diet.

The effect on yeast in the GI tract by dietary yeast (*S. cerevisiae* and *Wickerhamomyces anomalus*) was investigated by Huyben et al. (2017[a]). Diets where FM was replaced with 40–60% of yeast changed the microbiota in rainbow trout. In the diet with 60% *S. cerevisiae* and *W. anomalus* combined, *Candida albicans* increased, a potentially pathogenic yeast, and LAB decreased (Huyben et al. 2017[a]). The diet containing solely *S. cerevisiae* had lower effect on the microbiota in gut, both on yeast quantities and composition (Huyben et al. 2017[a]). However, Huyben et al. (2017[b]) showed that feeding live yeast to rainbow trout significantly increased yeast load, while increasing rearing temperatures had a negative effect on gut microbiota with lower yeast load and presence of LAB.

3. Material and method

3.1 Experimental setup

The experiment was carried out at the research station Aquaculture Centre North Inc. in Kälarne, Jämtland during a period of 2 weeks, with 2 additional weeks for acclimatization prior the introduction of dietary treatment. The fish species used throughout the experiment was Arctic charr of the strain Arctic superior, included in the Swedish breeding programme (Nilsson et al. 2010). The initial mean weight of the fish was 98.5 $\pm$ 19.8 g and the mean length, measured from the snout to the posterior end of the vertebra, was from start 19.4 $\pm$ 1.3 cm. The fish was reared in a flow-through system with rectangular fiberglass tanks with a capacity of 1 m^3 of water.

The fish were randomly divided into four different tanks two weeks before the experimental trial, in order to acclimatize to the new conditions. Each group consisted of 47 fish per tank. To obtain an adequate water depth (40 cm), the tanks were filled with 0.4 m^3 water, resulting in a fish density of 11.8 kg m^{-3}. The water temperature ranged between 6.2 – 6.3°C during the entire experimental period. The oxygen saturation was 96 percent (12.12 mg^{-L}) during the trial. The fish were fed on a commercial diet both prior to and during the reference sampling.

3.1.1 Experimental diets

The fish were fed either a control diet or three experimental diets in this study. The protein sources of the control diet (C) consisted of FM and soya, a formulation resembling to commercial diets (Lundh et al. 2014). In the experimental diets, 40% of commercial FM was substituted with *S. cerevisiae* (Y) or meal from blue mussel (M), respectively. The protein sources of BB consisted of 33% FM and 33% MM originated from the Baltic Sea, and 33% *S. cerevisiae*. Baltic Blend (BB) was produced by extrusion and lipid coating, see Olstorpe et al. (2014) for production method of feed. To conduct large quantities, commercially available *S. cerevisiae* grown on molasses was used. The chemical composition, gross energy and AA content of the diets are presented in table 1.

Table 1. Chemical composition (g kg^{-1} DM), gross energy content (MJ kg^{-1}) and amino acid content (g kg^{-1} DM) of the experimental diets

	Experimental diets[1]			
	C	Y	M	BB[2]
Crude protein	493	492	498	474
Sum of amino acids	439	491	466	408
Crude lipid	201	190	201	194
Ash	76	66	74	78
Gross energy	24.1	23.9	24.4	23.0
Essential amino acids				
Arginine	28.1	28.4	30.6	24.7
Histidine	11.0	12.1	10.4	8.8
Isoleucine	21.4	23.4	19.5	17.9
Leucine	36.4	38.6	35.7	29.1
Lysine	31.6	34.0	33.0	29.0
Methionine[3]	18.4	13.4	14.2	14.2
Phenylalanine	20.1	22.5	20.3	17.6
Threonine	19.5	20.7	20.7	17.2
Valine	26.3	28.6	23.9	21.7
Non-essential amino acids				
Alanine	25.3	26.6	24.9	21.5
Aspartic acid	43.1	46.0	45.2	37.8
Cysteine[4,5]	8.1	9.0	8.7	14.2
Glutamic acid	79.3	92.8	81.4	67.8
Glycine	24.4	25.4	25.9	23.8
Ornithine	0.0	2.3	3.2	6.2
Proline	22.4	26.6	25.0	21.6
Serine	17.4	20.7	23.1	18.8
Tyrosine[4]	6.7	19.8	19.6	16.4

[1] C = control diet, Y = diet with yeast (*Saccharomyces cerevisiae*), M = diet with blue mussel (*Mytilus edulis*), BB = Baltic blend diet

[2] Source: Data from Carlberg et al. 2018

[3] Amount present after oxidation of methionine to methionine sulphone

[4] Amount present after oxidation of cysteine and cystine to cysteic acid

[5] Conditionally indispensable (NRC 2011)

The total amount of feed available was approximately 2 kg per feed. The daily ration corresponded to 1% of the average initial weight of the fish (1g/fish/day). The feed ingredients of the experimental diets are presented in table 2. In all diets, titanium dioxide (TiO_2) was used as an internal digestibility marker.

Table 2. Feed ingredients (g kg^{-1}) of the experimental diets

	Experimental diets[1]			
Ingredients	C	Y	M	BB[2]
Fishmeal	468	281	280	-
Fish oil	89	92	89	71
Soy protein concentrate	36	28	36	-
Soybean meal	114	83	104	-
Rapeseed oil	35	34	32	47
Wheat gluten	34	60	39	50
Wheat meal	125	102	125	131
Titanium oxide	5	5	5	5
Mineral-vitamin premix	16	16	16	15
Cellulose	78	10	54	-
Mussel meal	-	-	220	212
Saccharomyces cerevisiae	-	289	-	253
Baltic Sea fishmeal	-	-	-	216

[1] C = control diet, Y = diet with yeast (*S. cerevisiae*), M = diet with blue mussel (*Mytilus edulis*), BB = Baltic blend diet

[2] Source: Data from Carlberg et al. 2018

For diet C, Y and M, the pellets size used was 2 mm. For the BB diet however, only 3 mm pellets were available. The 3 mm pellets were pestled by hand until it corresponded approximately the size of 2 mm pellets. The fish was fed continuously for 12 hours during the experimental period.

3.2 Sampling procedure

To determine the yeast flora in the fish gut, faecal samples were taken from four parts of the intestine: stomach (S), pylorus (P), mid intestine (MI) and distal intestine (DI). Initially, reference samples were collected from each experimental group (C, Y, M and BB), before providing the fish with either treatment. The reference sampling (T_0) was performed to get an overview of the normal yeast flora in the gut of fish fed a commercial diet. The reference sampling also provided an opportunity to examine the amount of available faeces in each intestinal part. Treatments were initiated after sampling procedure T_0 had been executed. Sampling procedures were subsequently executed one (T_1) respectively two (T_2) weeks after introduction to treatments. On each occasion, five individuals per tank were euthanized for collection of samples. Both weight and lenght measurements of fish were registered at the sampling occasions.

The fish was anesthetized by using tricaine methanesulfonate (MS-222) solution. A dose of 45 ml MS-222 was dissolved in five litres of water. To reduce

stress in fish, an equal amount of the basic compound, sodium hydrogen carbonate ($NaHCO^3$) was combined with MS-222. After approximately 15 minutes or when insensible, the fish was euthanized by a cut in the brainstem. Post mortem, the gastrointestinal tissues were removed and dissected. Ligatures were made between each gut section to prevent faeces from reposition. A sample of 0.05 g faeces was collected from each of the four parts of the gut: S, P, MI and DI. The different sections of the gut are displayed in fig. 1. For measurement of the DM content, an additional 0.5 g sample was collected. Due to lack of quantities of faeces available, the DM sample had to be pooled from the different gut sections (S, P, MI and DI).

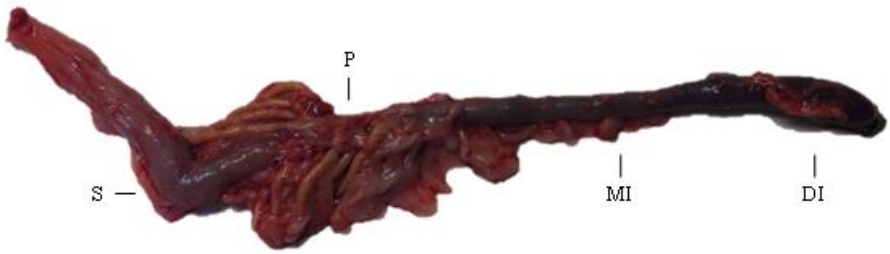


Fig. 1. The gastrointestinal tract of Arctic charr (Salvelinus alpinus) with the gut sections highlighted; stomach (S), pylorus (P), mid intestine (MI) and distal intestine (DI).

3.3 Analyses

3.3.1 Microbial sampling and quantification of yeast

The procedure for euthanizing and dissection were the same for all sampling occasions, however, there was a difference in dilution method between sampling time points. At the reference sampling (T_0), each fecal 0.05 g sample was directly distributed on a yeast peptone dextrose (YPD) agar plate (yeast extract, 9 g $liter^{-1}$, bacteriological peptone, 18 g $liter^{-1}$, D-glucose, 18 g $liter^{-1}$, agar 18 g $liter^{-1}$ and chloramphenicol 0.09 g $liter^{-1}$). The samples were evenly dispersed on the YPD plates using a sterile L-shaped cell spreader. The samples were incubated at 25°C during 48 to 72 hours. After incubation the colonies were counted and recalculated to CFU g^{-1} faeces.

At sampling T_1, the quantities of CFU of yeast were very dense. Calculations were performed in sections of the YPD agar plate, but in many cases the plates contained more CFUs than could be counted. Thus, the results from T_1 were not acknowledged further since the method of calculation CFU was considered inaccurate. Hence, no secondary agar plates were produced from sampling T_1.

At T_2, the samples were not directly distributed on YPD agar plates due to experience of increased cell counts at T_1. After dissection, a 0.05 g sample from each gut section (S, P, MI and DI) was placed in separate Eppendorf tubes®. The

faeces samples were diluted with 0.45 ml sterile peptone water (Bacteriological peptone 2 g liter^{-1}, Oxoid Ltd., Basingstoke, Hampshire, England), supplemented with 0.15 g liter^{-1} Tween 80 (Kebo AB, Stockholm, Sweden), and homogenized for 120 seconds. The homogenate was then serially diluted in peptone water and 50 µl was spread on to YPD agar plates and incubated at 25°C for 48 to 96 hours. For yeast counts, colonies were counted and multiplied by the dilution factor and expressed as CFU g^{-1} faeces.

3.3.2 Yeast identification

To obtain pure colonies for identification of yeasts, the colonies were re-streaked for isolation on a secondary YPD agar plate. From each primary YPD agar plate, a maximum of ten CFU were randomly chosen for isolation. The secondary YPD agar plates were incubated in 25°C for two to three days. The YPD plates were stored in a 2°C refrigerator after incubation.

3.3.3 DNA extraction and amplification

From each fecal sample, up to ten purified colonies was typed by polymerase chain reaction (PCR) amplification. Colonies were harvested with a sterile toothpick and resuspended in 20 µl 0.02 M NaOH, and by heating at 95°C for 10 minutes, cells were lysed. The PCR sample was mixed according to recommendations of puReTaq Ready-To-Go PCR Beads supplier (GE Healthcare, Buckinghamshire, UK).

Amplification of the D1-D2 region (approximately 600 bp) in the 28S rRNA gene was used for identification of yeast. Primers used were NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3'). The reaction process included a 2 min initial denaturation at 94°C followed by 35 cycles; denaturation 30 s at 94°C, annealing 30 s at 50°C, extension 2 min at 72°C, with a 5 min final extension step at 72°C. For electrophoresis, amplification products were transmitted to a 1% agarose gel in 0.5 Trisborate-EDTA (TBE) buffer. The settings for electrophoresis were 110 V cm^{-1} and 80 mA for approximately 60 min. Purification and sequencing of the samples were performed at Macrogen Inc (Amsterdam, The Netherlands). Sequence data files were compared against the National Center for Biotechnology Information (NCBI) database using nucleotide BLAST (http://www.ncbi.nlm.nih.gov). A positive match was defined as a sequence similarity of 99% of species existing in the database.

3.3.4 Qualitative and quantitative analysis of yeast in feed

Yeast quantification and identification of the experimental feeds and the feed ingredient FM was also performed. Triplicates of each feed were evaluated. The same protocol was used for quantification and identification as for analyse of the

yeast flora in faeces. A 1:10 serial dilution up to 10^5 was performed for each sample. The re-streaked colonies were incubated for four days and typed by PCR amplification with sequencing for the 28S rRNA gene.

3.3.5 Observations of bacterial growth and fungi

When monitoring the YPD plates, both bacterial growth and presence of moulds were registered. A visual observation and estimate of the bacterial growth were performed, when comparing the different YPD plates. Thus, no methods for quantification or identification of bacteria were used. Presence of moulds was also registered in terms of CFU and appearance. The fungi present at T_0 were identified through an external service. However, the moulds present at T_2 was never identified, hence no comparison between species found at the two sampling occasions was investigated further.

3.3.6 Statistical analyses

Mean values and standard deviations were calculated in Excel. To determine how the samples clustered and to find correlations between sampling, diet, gut segment, log CFU count data and yeast species data was analysed in a principal component analysis (PCA) model generated using Paleontological Statistics (PAST) version 4.11. One way ANOVA was performed in PAST to determine dietary effect on yeast load or composition, differences between gut sections or sampling occasion and on growth performance. Post-hoc test following significant results by ANOVA was done by Tukey's test for multiple comparison. The level of significance was $p<0.05$ for all statistical analysis.

4. Results

4.1 Fish growth

The mean initial body weights (IBW) and final body weights (FBW) for each group are presented in table 3 together with the mean initial lengths (IL) and final lengths (FL). Before introduction to the experimental diets, there were no differences in IBW or IL between fish. No significant differences were found between diets and FBW or FL after two weeks with the dietary treatments.

Table 3. Mean initial body weight (IBW), final body weight (FWB), initial length (IL), final length (FL) and standard deviation (of the fish included in the trial)

		Experimental diets[1]			
	p-value	C	Y	M	BB
IBW (g)	0.696	94.7 ± 16.5	103.8 ± 15.1	93.7 ± 14.8	98.7 ± 12.0
FBW (g)	0.535	132.7 ± 19.4	128.1 ± 27.0	133.5 ± 19.5	115.2 ± 20.2
IL (cm)	0.581	18.1 ± 0.9	18.6 ± 0.9	17.8 ± 0.8	18.2 ± 0.9
FL (cm)	0.281	19.9 ± 0.7	20.3 ± 1.3	20.2 ± 1.2	19.1 ± 0.9

[1] C = control diet, Y = diet with yeast (*Saccharomyces cerevisiae*), M = diet with blue mussel (*Mytilus edulis*), BB = Baltic blend diet

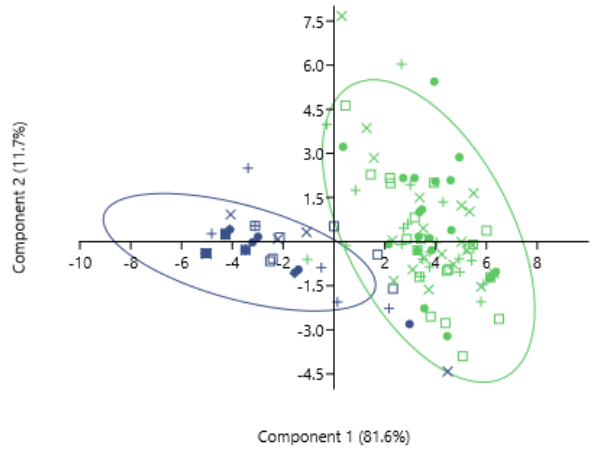
4.2 Analysis of gut yeast

4.2.1 Feed impact on gut yeast

Principal component analysis (PCA) of sampling time point, diet, gut segment, log CFU count and yeast species showed that there was a different clustering effect between sampling T_0 and T_2 (Fig. 2a). The variation of the data set in fig. 2a was explained by the first and second principal components (PC) with more than 93%. This indicates a correlation between sampling occasion, yeast load and dominant yeast species. No clear correlations or clustering patterns was shown by PCA between diets or gut segments when looking at both T_0 and T_2, indicating that no

clearly visible differences existed in yeast load or yeast flora composition that could be linked to these factors (Fig. 2b).

a)



b)

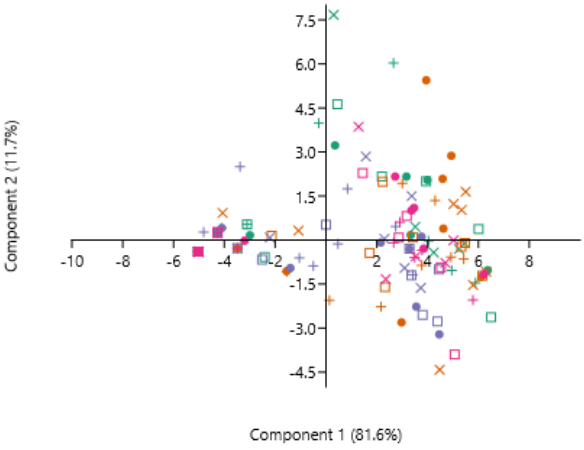
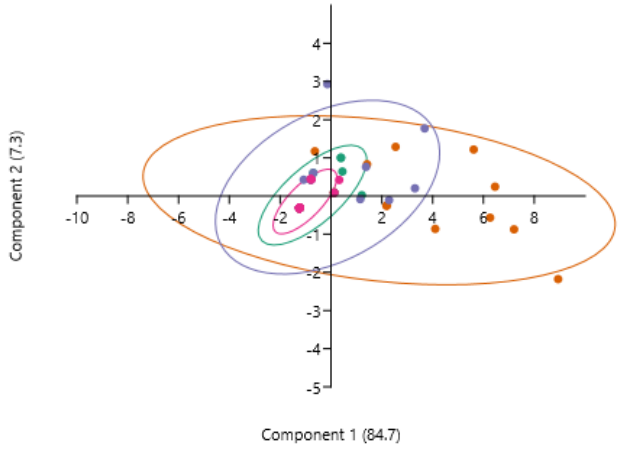


Fig. 2. Principal component analysis plots of yeast in the stomach (S: •), pylorus (P: +), mid intestine (MI: □) and distal intestine (DI: ×) in fish fed the experimental diets. The PCA shows ***a)*** *variation between the reference sampling (T_0: blue colour) and after two weeks of feeding with the experimental diets (T_2: green colour),* ***b)*** *variation between gut segments and the control diet (C: green colour), diets with 40% fishmeal replaced by Saccharomyces cerevisiae (Y: orange colour) or meal from blue mussel (M: purple colour) or the diet with a protein content consisting of 33% fishmeal, 33% S. cerevisiae and 33% meal from blue mussel (BB: pink colour) at T_0 and T_2.*

Analysing T_0 and T_2 separately (Fig. 3a and 3b) showed that samples clustered differently at the sampling occasions. However, no strong correlation or clustering pattern was displayed at T_0, except that the Y diet clustered differently compared to the other diets (Fig. 3a). At T_2, the Y and C diets clustered differently along the

second PC compared to the M diet, indicating a difference in yeast load and dominating yeast species between these diets (Fig. 3b). The first and second principal components also varied between T_0 and T_2, indicating that the variation in the data set also differed between before and after two weeks of feeding with the experimental diets.

a)



b)

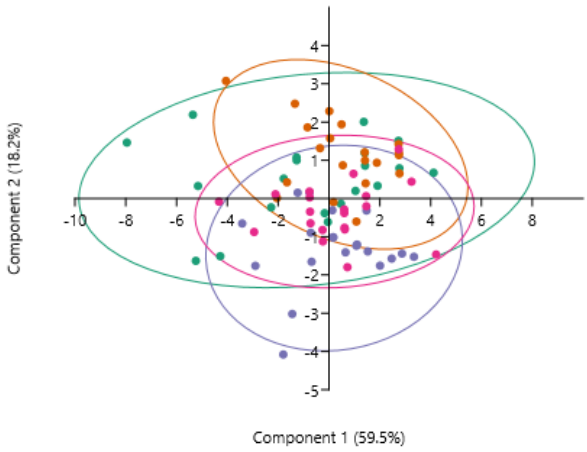


Fig. 3. Principal component analysis (PCA) plots of yeast in gut in the control diet (C: green colour), diets with 40% fishmeal replaced by Saccharomyces cerevisiae (Y: orange colour) or meal from blue mussel (M: purple colour) or the diet with a protein content consisting of 33% fishmeal, 33% S. cerevisiae and 33% meal from blue mussel (BB: pink colour). The PCA shows ***a)*** *variation between diets at the reference sampling (T_0),* ***b)*** *variation between diets two weeks after introduction to the experimental diets (T_2).*

Yeast load

After two weeks of feeding with the experimental diets, the yeast load in fish gut ranged between 4.1–7.5 log CFU g^{-1} (Fig. 4). Yeast colonies were found in all gut

segments except in the DI of fish in the BB group/tank at T_0. One missing value of CFU was registered for S in one fish fed the M diet due to non-countable colonies on the plates. Hence, the M diet mean value of CFU for S was based on four individuals. For all other mean values, n = 5.

The yeast load was significantly higher at T_2 than T_0 for all diets (p = 0.0009). At the reference sampling (T_0), the Y group/tank had a significantly higher yeast load than the other diets (C: p = 0.003, M: p = 0.016, BB: p = 0.0007). After two weeks of dietary treatment (T_2), the yeast load in the Y diet still was significantly higher compared to the other experimental diets (C: p = 0.024, M: p = 0.001, BB: p = 0.001). No other significant differences were found between diets and yeast load. No significant differences were found on yeast load between gut segments.

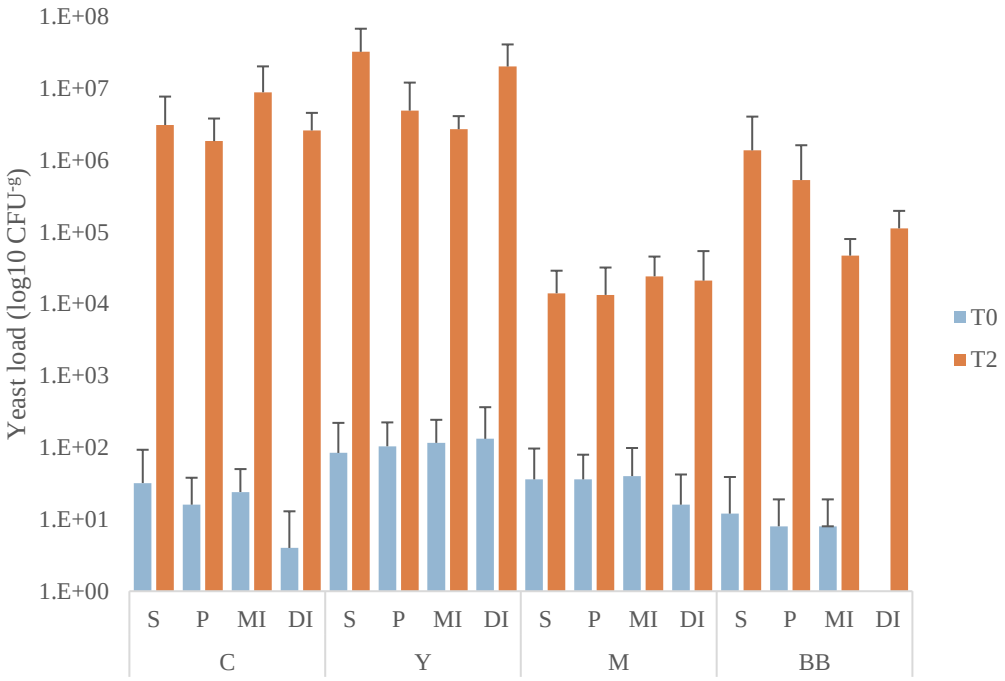


Fig. 4. Yeast load (mean ± SE) from different parts of the gastrointestinal tract (stomach (S), pylorus (P), mid intestine (MI) and distal intestine (DI)) plated on agar at reference sampling (T_0) and after two weeks of feeding (T_2) with the experimental diets (control (C), 40% of fishmeal replaced by yeast (Saccharomyces cerevisiae) (Y) or meal from blue mussel (M) and Baltic blend (BB)).

Yeast composition

In total, 13 different yeast species were found in all gut segments at the reference sampling (T_0) and after two weeks of feeding with the experimental diets (T_2) (Fig. 5). Between 68 to 70% of yeast isolates were identified as *D. hansenii* followed by *Debaryomyces sp.* (19-24%) and *Cryptococcus victoriae* (0-3%). *Debaryomyces hansenii* was present in all diets and gut segments except in group/tank C (S) and BB (MI and DI) at T_0. No significant differences were found between gut segments or diets for *D. hansenii*. However, *D. hansenii* was significantly more abundant at

sampling T_2 than at T_0. The sampling time point also had a significant effect on *Debaryomyces sp.* with higher abundance at T_2. *Saccharomyces cerevisiae* was only detected in fish fed BB at T_2 (MI). The BB group/tank had a slightly different yeast profile at T_0 compared to the other diets and no CFU identified in DI. When monitoring YPD plates, red yeast was found solely in the Y diet at T_2 e.g., the diet containing *S. cerevisiae*.

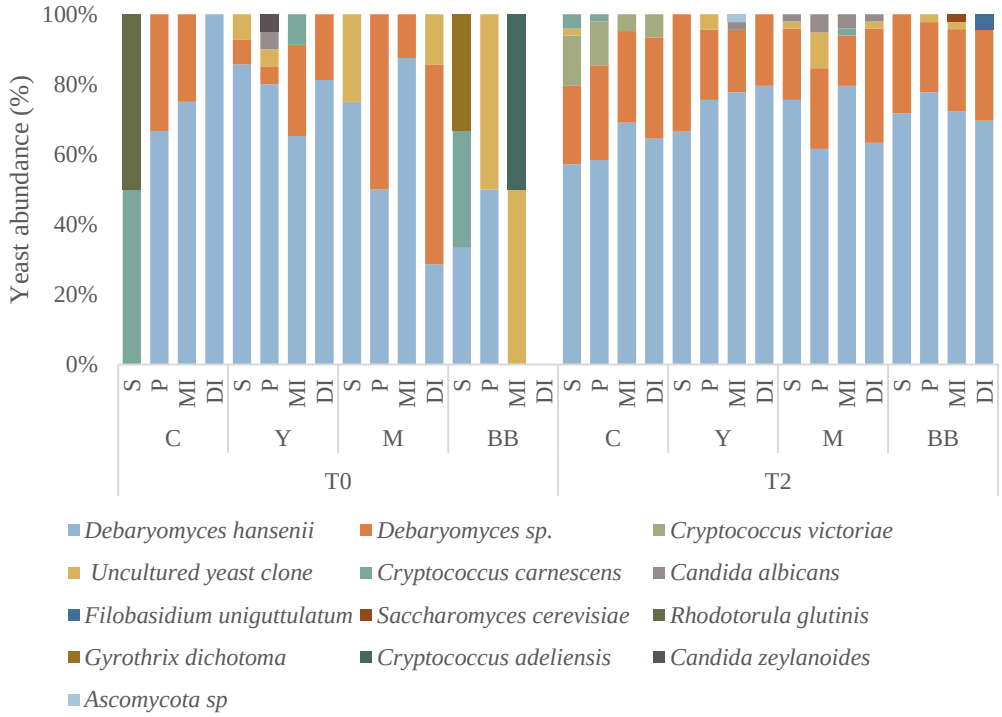


Fig. 5. Yeast abundance of selected colonies sequenced for the 28S rRNA gene in the different gut segments (stomach (S), pylorus (P), mid intestine (MI) and distal intestine (DI)) of fish at the reference sampling (T_0) and after two weeks (T_2) of feeding with the experimental diets (control (C), 40% of fishmeal replaced by yeast (S. cerevisiae) (Y) or meal from blue mussel (M) and Baltic blend (BB)).

4.2.2 Yeast in feed

The results of quantification and identification of yeast in the experimental feeds, and in the commercial FM ingredient, are presented in fig. 6a and 6b. The largest quantities of yeast were found in the feed containing yeast (diet Y) followed by the control feed (C) (Fig. 6a). The feed including MM (diet M) contained the lowest number of yeasts CFU g^{-1}.

In all feeds, *D. hansenii* was present (Fig. 6b). The species *S. cerevisiae* was found in all feeds except the C feed. In the feeds M and BB, *S. cerevisiae* represented approximately 42% of the total CFU found. The Y feed contained less than 10% of *S. cerevisiae*. Commercial FM was included as a feed ingredient in the C, Y and M feeds. The dominant yeast found in FM was *Cryptococcus* (75%). The

yeast species found in FM (*Cryptococcus*, *Sporobolomyces ruberrimus* and *Rhodotorula mucilaginosa*) differed from the yeast species found in the feeds.

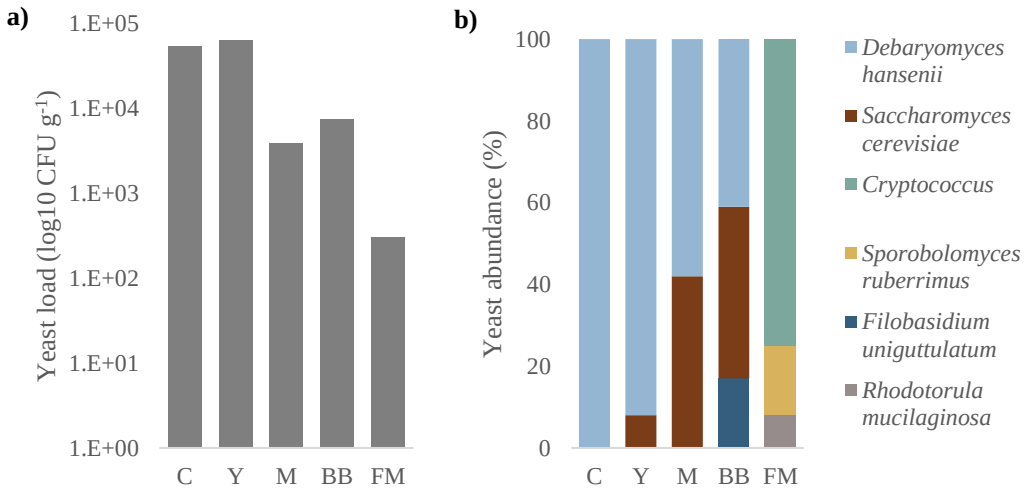


Fig. 6. Yeast present in the experimental feeds; control (C), 40% of fishmeal replaced by yeast (S. cerevisiae) (Y) or meal from blue mussel (M), Baltic blend (BB) and in the raw material fishmeal (FM). ***a)*** *Yeast load plated on agar and,* ***b)*** *Yeast abundance of selected colonies sequenced for the 28S rRNA gene.*

4.2.3 Bacteria and fungi

Bacterial growth

In all diets, bacterial growth was registered when monitoring the YPD plates. The greatest quantities of bacterial growth were found in fish fed the M diet followed by the Y diet. Samples from fish fed the BB diet also contained bacteria however, not to the same extent. The smallest quantities of bacteria were found in fish fed the C diet. Bacteria existed in all gut sections (S, P, MI and DI) in the M and BB diet. In the C and Y diet, bacterial growth was found in all gut sections except in the stomach (S).

Moulds

When monitoring the YPD plates, growth of moulds was registered in all diets at both T_0 and T_2. However, not all gut segments contained moulds. At both sampling occasions, the largest number of moulds were found in fish fed the BB diet. At both sampling occasions, the M diet had the lowest quantities of moulds. In the M diet, moulds were only found in the DI at the reference sampling (T_0), and in the S and P at T_2. In the Y diet, mould growth was absent in the S. All other gut segments contained growth of moulds.

5. Discussion

This study investigated the effect of the different feeds (C, Y, M and BB) based on growth and effect on the microbial population, with a focus on yeasts, in different parts of the GI tract in Arctic charr. Yeast, as a part of the microbiota in gut, plays an important role of various functions in the body (Berg 1996; Romero et al. 2014). Relatively small populations of yeast (<1% of the total microbial isolates) present in the gut can affect the host, as yeast has a large cell volume compared to e.g., bacteria (Gatesoupe 2007). Depending on yeast characteristics, it may also affect the host differently as some yeast have positive health effects and some might be pathogenic (Gatesoupe 2007). The main result of this study was some differences in the amount of yeast linked to diet, and that there was consistently a lot of *D. hansenii* in the samples. The high abundance of *D. hansenii* in all diets both before and after introduction of dietary treatments corresponds with previous results (Aubin et al. 2005; Gatesoupe 2007; Huyben et al. 2017[a]). Huyben et al. (2017[a]) found that *D. hansenii* represented between 84 to 90% of the yeasts found in the gut of rainbow trout fed diets with FM substituted by 20, 40 and 60% of *S. cerevisiae* or *W. anomalus.* In agreement, Andlid et al. (1995) showed that over 95% of yeast isolates in rainbow trout fed both a commercial FM diet or a diet including yeast, were identified as *D. hansenii* or *S. cerevisiae*. The findings in the present study suggest that *D. hansenii* might constitute a large portion of the yeast flora in Arctic charr as well as in rainbow trout (Andlid et al. 1995; Gatesoupe 2007; Huyben et al. 2017[a]). *Debaryomyces hansenii* might also have been spread in the environment and *D. hansenii* was also present in the feeds, which could explain why the amount of yeast increased during the course of the study. Between 19 to 24% of the isolates in the present study were identified as *Debaryomyces sp.* and it may be possible that some of these colonies would be classified as *D. hansenii* if investigated further, which could result in up to 87-94% of isolates in the study being *D. hansenii*.

The low abundance of *S. cerevisiae*, particularly for the group feed the yeast diet, was somewhat surprising. In a previous study, little to no abundance of *S. cerevisiae* was found in gut of rainbow trout after inclusion of *S. cerevisiae* or *W. anomalus* in diet (Huyben et al. 2017[a]). Also, Aubin et al. (2005) did not retrieve *S. cerevisiae* in the microbiota when feeding *S. cerevisiae* as a probiotic in feed. These findings

are contradicted by results from another study, where 100% of yeast isolates in rainbow trout gut consisted of *S. cerevisiae*, after feeding a yeast diet with 40% of FM replaced by *S. cerevisiae* (Huyben et al. 2017[b]). However, the yeast diets in the previous studies differed from each other, and in parts from the present study, with varying yeast inclusion levels and diverse feed manufacturing processes. Extruded feed was used in the present study as well as in the study by Huyben et al. (2017[a]) with the low levels of *S. cerevisiae* in gut, indicating that the heat and pressure treatment of the feed probably contributed to inactivated yeast (Huyben et al. 2017[a]). However, the diet formulation in the present study (40% FM replaced by *S. cerevisiae*) resembled to the yeast inclusion levels in the previous study with the high abundance of *S. cerevisiae* in gut, where live yeast was used in feed (Huyben et al. 2017[b]). The different outcome might be explained by the feed processing, as cold pelleted feed results in higher life yeast quantities than extruded feed (Huyben et al. 2017[a]).

The results showed that the Y feed had the lowest level of *S. cerevisiae*, with the exception for the C feed. The most unexpected result was that the M feed contained *S. cerevisiae* to the same extent as the BB feed and considerably more than the Y feed. This despite that *S. cerevisiae* was added to both the Y and BB feed. No explanation for the presence of *S. cerevisiae* in the M feed was found. In the study by Huyben et al. (2017[b]), 100% of the relative abundance of live yeast in the yeast feed was identified as *S. cerevisiae*. In this study, the yeast composition in feed was dominated by *D. hansenii* with *S. cerevisiae* present to some extent in all feeds except the C feed. *Filobasidium uniguttulatum* was also found in the BB feed. Thus, there were various yeasts that grew in the feed and that were added as a feed ingredient. The yeast load found in the gut is influenced by the amount of live yeast fed to the fish and the processing of feed can affect the levels of live yeasts in the feed (Huyben et al. 2017[b]). The Y feed used in the present study contained a yeast load of 4.8 log CFU g^{-1} where 92% of the yeast isolates consisted of *D. hansenii*. In an extruded feed with 40% FM substituted by yeast, Huyben et al. (2017[a]) observed a yeast load of 2.4 log CFU g^{-1} where the dominating yeast species was *S. cerevisiae* with minor levels of *Saccharomyces roseus*. In comparison, a cold-pelleted yeast feed in another study resulted in a higher (7.6 CFU g^{-1}) yeast load than the extruded feed and with 100% of live yeast isolates identified as *S. cerevisiae* (Huyben et al. 2017[b]). In the present study, the low to no abundance of *S. cerevisiae* in feed, indicate that yeast was inactivated by feed processing (Huyben et al. 2017[a]).

The CFU count can also be affected depending on methodology and on levels of inactivated yeast (Huyben et al. 2017[b]). A yeast load of 7.6 ± 6.2 log CFU g^{-1} was calculated by Huyben et al. (2017[b]) when using the agar plating method for live yeast CFU count in feed where 40% of the FM was substituted by *S. cerevisiae*. In comparison, when using chamber counting of cell counts in the same study, the

yeast diet recorded a yeast load of 9.7 ± 7.6 log cell counts g^{-1} (Huyben et al. 2017[b]). The chamber counting methodology is described by Huyben et al. (2017[a]). In short, the yeast is stained and viewed in a light microscope in magnification. The method also enables to count yeast viability as the viable cells are unstained (Huyben et al. 2017[a]). As the present study used CFU count directly on agar plates, yeast load might have differed compared to if the chamber counting method would have been used. Also, it would have been possible to identify cell viability and to differentiate between viable and inactivated yeast.

The yeast load in fish gut ranged between 0–2 log CFU g^{-1} at the reference sampling with a significant increase to 4–7 log CFU g^{-1} two weeks after introduction to the experimental diets. For the BB group, the DI at the reference sampling had no viable CFU, reported as a missing value, which might have influenced the results. The increase in yeast load might depend on the GI tract of fish being suggested as a suitable reproductive site for yeast (Andlid et al. 1995). Also, the feeds used in this study contained levels of yeast which might have had an impact in the yeast load in gut. Andlid et al. (1995) found an increase from 3 to 9 log CFU g^{-1} in yeast load when feeding *D. hansenii* and *S. cerevisiae* to rainbow trout. Possible explanations to this increased yeast load in gut, both in the previous study as in the present study, might be that the yeast grows in the faeces or the intestine, or that the yeast, being adhesive, is retained and concentrated in the gut (Andlid et al. 1995). After six weeks of dietary treatment with *S. cerevisiae* in cold water (11°C), Huyben et al. (2017[b]) found a yeast load in gut of rainbow trout of 7.4 ± 7.0 log CFU g^{-1}, resembling to the result in the present study.

The yeast load was significantly higher in the Y diet compared to the other experimental diets at both T_0 and T_2. The hypothesis of this study was that fish fed with *S. cerevisiae* would have a higher number of CFU in faeces compared with the other treatments. Huyben et al. (2017[a]) found a significant increase of yeast load between a yeast diet with 40% FM replaced by *S. cerevisiae* compared to a FM diet fed to rainbow trout. However, in the present study the Y diet already had a higher yeast load compared to the other diets at the reference sampling (T_0), when all fish were fed a commercial diet. At T_0, the fish were divided and acclimatized in different tanks, but no dietary treatment had been initiated. Hence, the environment might have influenced the result. The method in this experiment did not have an optimal design for statistical analysis since only one tank per diet were used. Instead, triplicates of each experimental diets should have been used, including tank as a factor in the statistical model (Vidakovic et al. 2016; Huyben et al. 2017[a]). With the present study design, it was not possible to investigate further if the environment might have had an effect on the result.

No differences between gut segments were found regarding yeast load. Nyman et al. (2017) did not find any differences in microbiota composition between the proximal and distal gut, when studying bacteria after feeding *S. cerevisiae*, *R.*

oryzae and MM diets to Arctic charr. Further research on the effect of dietary *S. cerevisiae* on both yeast load and composition in Arctic charr is needed to clarify indications found in this study.

There were no significant differences in weight or length between the experimental groups after two weeks. In agreement, Vidakovic et al. (2016) found that Arctic charr fed diets with intact *S. cerevisiae,* blue mussels or a reference diet did not significantly differ in weight gain after a period of 99 days. The weight gain for this experiment was not possible to calculate since the fish were slaughtered at sampling.

Huyben et al. (2017[a]) was the first study to identify *C. zeylanoides* and *C. carnescens* in gut of rainbow trout. Both these yeast species were identified at the reference sampling and *C. carnescens* was also found in fish fed the C and M diet after two weeks of dietary treatment. As minor components of microbiota in rainbow trout, *Candida sp*. and *Cryptococcus sp.* has been mentioned (Gatesoupe 2007). The method used in this, and other studies has been dependent on the yeast being viable (Huyben et al. 2017[a]; Huyben et al. 2017[b]). In addition, the number of colonies identified has been quite limited. Further studies combined with usage of refined methodology for yeast sequencing precision are likely to result in discovering new species in the gut of salmonid fish in the future. In the M diet, the pathogenic yeast *C. albicans* occurred after two weeks of feeding with the experimental diets. The *C. albicans* is a part of the normal microbiota in fish (Huyben et al. 2017[a]). Huyben et al. (2017[a]) discovered a significant increase of *C. albicans* with higher inclusion of yeast in diet (60% *W. anomalus*), which might have been the result of microbial imbalance or dysbiosis.

The feed ingredient FM contained 2.4 log CFU^{-g} in yeast load composed of *Cryptococcus, S. ruberrimus* and *R. mucilaginosa*. Similar findings were found by Huyben et al. (2017[a]) when analysing FM, with the difference that *D. hansenii* and *S. cerevisiae* also were detected in FM. The yeast composition in FM differed from the yeast species found in the feeds that contained FM. One possible explanation is that the feeds and FM probably contained other species, but as serial dilution was used to be able to count CFU on agar plates, these species did not appear on the plates (Huyben et al. 2017[b]).

Both growth of bacteria and mould was estimated visually when monitoring the agar plates, however not resulting in any reliable data. The diets containing most bacteria was the M and Y diet. Since no analysis on bacteria was made on the feed, it was not possible to know if these two feeds contained more bacteria than the C and BB feed. Also, the different feeds could have contained different types of bacteria, of which some survived better in fish intestine and/or on the agar plates than others. Moulds were found in all diets at both sampling occasions, with the highest presence in fish fed the BB diet. The agar plates used was intended for growth of yeast, meaning that the conditions were not optimal for growth of bacteria

or moulds, and if analysed, might not have given a representative image of the flora. To draw more conclusions from the growth of moulds, identification of the fungi at the second sampling occasion would have been necessary for comparison with the results from the reference sampling.

6. Conclusion

In conclusion, the study showed that there were differences in the amount of yeast in gut linked to diets that were evaluated, with a significantly higher yeast load in fish feed the diet including *S. cerevisiae* (Y). No differences were found in yeast composition between diets or between gut segments. *Debaryomyces hansenii* was the dominant yeast species found in gut regardless of diet type. Differences of amount of yeast could also be linked to time (i.e., before and after). No differences were found on growth performance in fish between diets. Further research on the effect of yeast in feed is necessary for continued understanding of the impact on the yeast flora of Arctic charr.

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Popular science summary

Aquaculture is a rapid growing sector and an important contributor to the world food supply. Historically, the aquaculture industry has been dependent on marine resources as fishmeal and fish oil for protein sources in feed for farmed fish. Due to unreliable catches, the threat of overfishing and fluctuations in price, the industry itself has started to look for alternatives to fishmeal and fish oil to enhance sustainability within aquaculture. When looking for replacements for these marine resources, it is of great importance to consider a suitable amino acid profile and protein content for fish. From a sustainability perspective, the most favourable option would be to use feed ingredients not suitable for direct human consumption to avoid competition of feed resources. The protein rich blue mussel has been discussed as a substitute to fishmeal for a long time, where mussels below marketing size would be ideal to use for this purpose. Plant protein is commonly used as an alternative to marine resources in aquaculture, however plant protein can many times be directly consumed by humans. Instead, it might be necessary to look further down the food chain. Microorganisms, such as yeast and fungi, has been of interest as a protein replacer in fish feed, where the baker's yeast (*Saccharomyces cerevisiae*) has been suggested as a suitable option with desirable traits.

All feed intake by the fish need to pass through the gut. In the fish gut, there is a complex system of microorganisms inhabiting the surfaces, referred to as the microbiota. The microbiota affects various important processes of the host. Yeast, as being one of the types of microorganisms present in fish gut, can exist in large number and in various composition. The normal microbiota is a dynamic system but can be seen as a base of the microorganisms inhabiting the gut in normal conditions. What happens to the yeast flora when fish are feed yeast compared to other diets? Will the diet alter the number of, or the diversity of yeast species found in the gut? Will different gut sections respond differently to the feed? These were all questions that were addressed in the present study. Four different diets were fed to Arctic charr for two weeks. A reference diet resembled to a commercial diet, containing fishmeal as a protein source was used. In two of the experimental diets, 40 percent of the fishmeal was replaced by baker's yeast or by blue mussel meal. The third experimental diet contained ingredients originated from the Baltic Sea, with a protein content of 1/3 fishmeal and 1/3 blue mussel meal combined with 1/3 baker's yeast. Four different parts of the gut were analysed to see if the response

differed between these sections in the gastrointestinal tract. Differences in the yeast flora composition and yeast loads from before and after two weeks of dietary treatment was investigated together with fish growth performance. Also, bacterial and fungi growth was estimated when analysing the samples. The yeast load and yeast composition in the feeds were also analysed. No differences in growth performance were found between the different groups at the end of the study. Regarding the yeast flora, there were no differences between the yeast species found in the gut between the different diets. *Debaryomyces hansenii* was the dominate yeast species throughout all samples in the experiment i.e., not the same yeast species that was added in the feeds. The gut sections did not have an effect on the number of, or the diversity of yeast species in this experiment. However, when looking at the number of yeasts found in gut, there were indications pointing towards that the fish fed the diet containing 40 percent of baker's yeast, had a higher number of yeast present in the gut than the other diets. However, the study design was not optimally designed for this type of analysis and therefore the results need to be looked at as just indications. Further research needs to investigate whether feeding baker's yeast to Arctic charr results in higher yeast amounts in gut.

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