Evaluation of natural astaxanthin produced by microalgae as a potential pigment source for Atlantic salmon (*Salmo salar*) feed

Why: Cultured salmon (*Salmo salar*) is an important carnivorous fish with over 2.3 million metric tons produced in 2015 (FAO 2017). The pink to red color of the fillet is one of the most important quality criteria for salmon (Sigurgisladottir et al. 1997). This pigmentation is due to deposition of carotenoids in the muscle of salmon, mainly astaxanthin and to a lesser degree canthaxanthin, in farmed salmon (Storebakken and No 1992). Since salmon is unable to synthesize astaxanthin, the carotenoids originate from micro-algae in the wild, or are synthetically produced for inclusion in the diets of cultured fish. These include engineered tomatoes, carrots and microorganisms as alternative sources of astaxanthin (Henke et al., 2016; Lu et al., 2017). Increasing consumer demand and development of cultured salmon markets calls for expansion of sources of astaxanthin, and by methods acceptable to consumers, and in forms easily bioaccessible by salmon. Synthetically produced astaxanthin are often extracted for inclusion in the diet. This can be laborious, costly and with limited efficiency. There is the need therefore to produce astaxanthin-contained feed ingredient devoid of an extraction step before inclusion in the diet. It has been shown that some microalgae with high levels of astaxanthin can be good candidates of feed ingredient in salmon diet (Lorenz and Cyseswki, 2000; Zhou et al., 2018).

Getting Atlantic salmon to deposit algal astaxanthin in their muscle tissue has barriers to overcome. Only 5 to 12% of the synthetic astaxanthin fed to the fish is retained in the muscle tissue with most of the carotenoid either being metabolized or excreted (Wathne et al. 1998, Aas et al. 1999). Atlantic salmon cultured at 12ºC had 10% greater astaxanthin apparent digestibility coefficient compared to fish at 8ºC. When natural astaxanthin from *Phaffa rhodozyma* (yeast) were compared to the synthetic astaxanthin in salmon diets, Atlantic salmon could access the natural astaxanthin in the yeast cells and had a higher retention in the muscle compared to the synthetic astaxanthin (Bjerkeng et al. 2007). This indicates that the astaxanthin in microalgal cells could be accessible to the fish. One approach to improve the stability of astaxanthin during storage and after ingestion, and ensure its targeted delivery include encapsulation (Zu et a., 2018). It involves the entrapment of the material into porous gels among other immobilization techniques. The objectives of this study are;

What: The purpose of this proposal is to evaluate the stability of astaxanthin in microalgal cells (McaAst) and to determine if the astaxanthin can potentially be used by Atlantic salmon. Encapsulating the microalgal cells will increase stability of the astaxanthin.

Where: National Cold Water Marine Aquaculture Center, Franklin Maine; Bigelow Laboratory, Booth Bay Harbor Maine and Delaware State University, Dover Delaware.

Who: Dr. Gary Burr, National Cold Water Marine Aquaculture Center; Dr. Alberta Aryee, Delaware State University. Dr. Aryee will carry out the encapsulation, *in vitro* digestion and bioaccessibility studies, while Dr. Burr will carry out the stability evaluation and *in vivo* digestibility studies. Dr. Michael Lomas will provide the microalgae.

How: (1) Encapsulation: Bigelow Laboratory supplied with the microalgae containing astaxanthin (McaAst). McaAst was encapsulated or freeze dried and stored in a -80 °C freezer until the encapsulation process has been completed. McaAst was encapsulated in a food-grade hydrocolloid, sodium alginate (NaAlg) by ionotropic gelation techniques. About 1 - 5% NaAlg was dispersed in milliQ water at room temperature under vigorous magnetic stirring. McaAst at 1 - 3% was added to the dispersed NaAlg and further mixed. The blended dispersion of NaAlg and McaAst was introduced in 20 mL plastic syringes and pumped into 0.1 M calcium chloride solution under low magnetic stirring. The microcapsulated McaAst waswashed and dried at room temperature. Encapsulation efficiency (EE) of McaAst in NaAlg calculated. Scanning electron microscopy (SEM), confocal laser scanning microscopy (Fig. 1) and particle size analysis was used to study the morphology and size of the microcapsules. *In vitro* release study of encapsulated McaAst was determined per the method described by Llorens et al. (2015) with slight modification. In brief, McaAst microcapsules was incubated in PBS (0.01 M, pH 7.4) (release media) at 37°C and under shaking at 100 rpm. Aliquots of the release media was withdrawn at 15, 30, 60, 90 and 120 min and absorbance read at 455 nm. **(2) Stability Test:** Triplicate freeze-dried McaAst samples were stored in a cool room used to store fish feed for 8 weeks. Samples were freeze dried McaAst, (reference), 1% NaAlg + McaAst, 2% NaAlg + McaAst, and 3% NaAlg + McaAst. Samples were taken every week and analyzed in duplicate using high pressure liquid chromatography (HPLC) to determine the stability of the astaxanthin. Currently HPLC analysis is the best way to evaluate the amount of astaxanthin in salmon muscle tissue and algal cells (Christiansen et al. 1995; Ljungqvist et al. 2012). **(3) *In vitro* Digestibility study:** *In vitro* digestibility study will be used to predict accessibilty encapsulated McaAst. The two treatments that show the greatest stability in the previous test was used in this study along with the reference McaAst. The method described by Rungruangsak-Torrissen et al. (2002) with slight modification was used, that is instead of determining the amino acid reactive groups, astaxanthin content was measured. The samples after digestion was treated as described by Zhou et al. (2018). **(4) HPLC analysis**: Astaxanthin content of salmon fillet was determined using HPLC per the method described by Ljungqvist et al. (2012). Standards were purchased from Chromadex (Irvine, CA). **(5) *In vivo study:*** A digestibility study was conducted using 500 g or greater Atlantic salmon at the USDA ARS National Cold Water Marine Aquaculture Center in Franklin, ME. About 180 g was stocked in nine 1.0 m^3 tanks (20 fish per tank depending on size) and supplied with 4-L min^{-1} of oxygen saturated water at a temperature of 13°C to14°C from a recirculating biological filtration system (32 ppt salinity). The fish were fed diets containing 60 ppm astaxanthin either as an encapsulated product, freeze-dried algal product (product that had greatest in vitro digestibility), or synthetic astaxanthin. The diets were manufactured using commercial equipment and methods. The fish were manually stripped of feces and the feces from each tank pooled until enough dried feces is obtained to weigh 5 g. The feces and diets will then be analyzed for astaxanthin content (described above). The methods of Cho et al. (1982) and Bureau et al. (1999) was used to estimate apparent digestibility coefficients (ADCs). Yttrium oxide will serve as the inert maker. Apparent digestibility coefficients of astaxanthin in the test diet. Samples were sent to the Bozeman Fish Technology Center for Yttrium

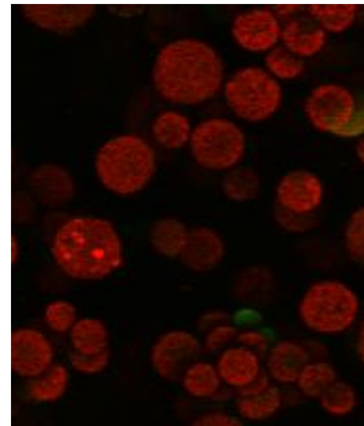

Figure 1: Confocal laser scanning microscope image of microcapsule prepared by ionotropic gelation method containing microalgae astaxanthin (McaAst).

analysis. The amount of astaxanthin contained in the feces will determine accessibility of the compound to the fish.

Results and Discussion:

Due to pandemic restrictions the in vitro analysis has yet to be done but will be completed by the end of the calendar year.

The microalgae was encapsulated with three different concentrations of sodium alginate, 1%, 2% or 3% and the astaxanthin was added at various concentrations (1, 2, 3%) for testing. After encapsulation, the samples were heated to 100℃ for 15 minutes to simulate the heat of extrusion and then stored in a cool, dry feed room for the duration of the stability study. Samples containing higher alginate levels appeared to have better storage characteristics (Figure 1). Samples containing higher levels of astaxanthin appears to have lower retention(29-54% retention) compared to samples with 1% astaxanthin (82-90% retention) the 1% alginate samples appeared to suffer from oxidation and the freeze dried algae also appeared to store very well with a high astaxanthin retention (75%). This indicates that incorporating lower astaxanthin levels into the encapsulate product will increase stability. Storing the freeze dried sample in an capped tubed also appears to reduce oxidation of astaxanthin.

The digestibility results were similar to other studies with ADC values ranging from 52-62%) (Bjerking et al. 2007; Ytrestøyl et al. 2007). These values match the literature where only 5-12% of the astaxanthin was incorporated into the fillet of Atlantic salmon. This study indicates the microalgae and encapsulated microalgae is a potential pigment source for Atlantic salmon feeds. Further studies are need to measure uptake and deposition in the fillet.

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Table 1. Encapsulation of microalgae for studies both stability test and digestibility test.

Key		
Sample	% Alginate	% Asta
1	1	1
2	1	2
3	1	3
4	2	1
5	2	2
6	2	3
7	3	1
8	3	2
9	3	3
10	Algae	

Figure 1.

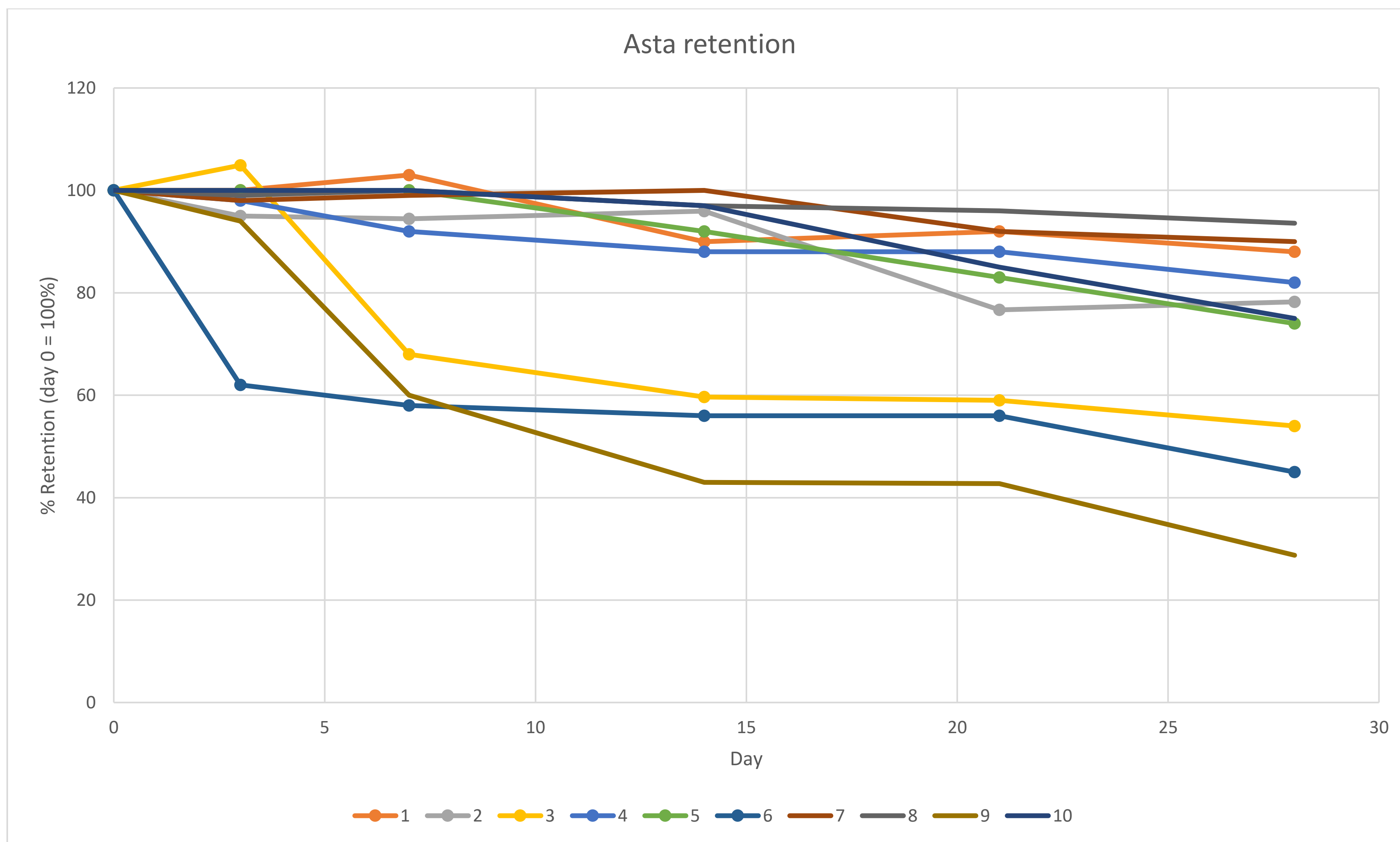

Figure 2.