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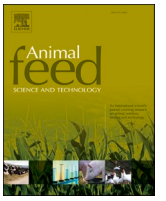

# The use of tunicate (*Ciona intestinalis*) as a sustainable protein source in fish feed – Effects on the extrusion process, physical pellet quality and microstructure

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ABSTRACT

The aim of this research was to study the effect of the replacement of fishmeal (FM) or soy protein concentrate (SPC) by tunicate meal (*Ciona intestinalis*) on the extrusion process, physical pellet quality, pellet expansion, and microstructure. The experiment was based on a 3-component simplex-centroid mixture design. Models with $R^2$ in the range of 0.574–0.999 (P = 0.03 to < 0.0001) were established for specific mechanical energy (SME), temperature behind extruder die ($T_{die}$), apparent dough viscosity in the extruder die ($V_{die}$), pellet hardness, durability, expansion, and microstructure parameters. Increase in SME and $T_{die}$ were mainly explained by the reduced lipid content in the feed mix due to the replacement of FM with tunicate or SPC. Reduced $V_{die}$ was mainly an effect of increased tunicate level and with the lowest value found for the pure tunicate blend. There was an increase in volumetric expansion and open porosity by replacement of SPC with tunicate, explained by this decrease in $V_{die}$. Reduced hardness and durability were found for blends high in SPC due to incomplete plasticisation. Extrudate expansion, fat adsorption capacity and leakage were explained by the extrudate microstructure measured by X-ray micro-tomography. Within the boundary of the chosen design, 64% of the FM can be replaced by tunicate meal without compromising physical feed quality. Due to its viscosity behaviour, tunicate meal can improve pellet expansion and fat absorption capacity of extruded feed.

## 1. Introduction

Searching for new protein resources that can replace fishmeal and plant-based ingredients are vital to secure a sustainable growth of the aquaculture industry. This can be other marine resources, algae, single-cell, marine and terrestrial animal by-products and insects (Tacon and Metian, 2008; Sørensen et al., 2011; Tacon et al., 2011). During the development process from new raw materials to protein ingredients it is highly important to study their technical properties and nutritional constraints, since both determine possible inclusion levels in fish feed.

Commercial fish feed is most commonly produced by use of extrusion technology and studies have documented that processability

*Abbreviations:* CGM, corn gluten meal; Faba, horse beans; FM, fishmeal; MCS, mean cell size; SEI, sectional expansion index; SME, specific mechanical energy; SPC, soy protein concentrate; $T_{die}$, temperature behind extruder die; Tunicate, meal of *Ciona intestinalis*; $V_{die}$, apparent dough viscosity in the extruder die; WG, vital wheat gluten; WSI, water stability index.

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and end product characteristics are dependent on the physicochemical and rheological properties of the feed ingredients (Sørensen, 2012; Samuelsen et al., 2013, 2014, 2018; Samuelsen and Oterhals, 2016). If an ingredient gives reduced durability and/or expansion and/or increased oil leakage this will limit the amount possible to use in commercial feeds. In the extrusion process the ingredients are added water, heated by steam in a preconditioner and by viscous heat dissipation through mechanical mixing in an extruder barrel. During processing, the powder is unfolded to a flowable material with reduced viscosity. This plasticized dough can then be shaped and expanded through the extruder dies creating extrudates with new intramolecular binding networks (Mercier et al., 1989). Lipids lubricate the extruder screws and poor physical feed quality, and reduced expansion can be a consequence if the lipid level exceed 120 g/kg (Rokey, 1994). For high-energy salmon feed most of the lipids therefore have to be added and adsorbed into the dried feed in a vacuum coating process after drying (Strauch, 2005). Pellet durability, oil adsorption capacity and oil leakage during storage are directly linked to the degree of expansion and the microstructure parameters such as open and closed porosity, pore size distribution, cell wall thickness and interconnectivity between the pores (Draganovic et al., 2013, 2019; Dethlefsen, 2017). An ideal product with controlled expansion should have evenly sized small pores connected with each other and the surface. Smaller pores will have high capillary pressure than larger pores and thereby high potential of oil uptake and retention (Moreira et al., 1997).

Recently, the nutritional constraints of Tunicate (*Ciona intestinalis*) meal in feeds, have been studied for Atlantic salmon. At a dietary level of approximately 17%, substituting 50% of dietary fish meal protein promising results in terms of growth, biometrics, fillet and liver total lipids and fatty acid profile and tissue morphology were observed (Kousoulaki et al., 2020). Apart from vanadium (V) which was found at higher levels in tunicate, substituting fish meal with tunicate meal reduced the levels of contaminants, toxins, and heavy metals in salmon whole bodies (e.g. toxaphene and arsenic). The dry tunicate meal used in the study contained relatively low levels of protein (ca. 41%) and lipids (ca. 3%), but it was high in arachidonic acid, EPA and DHA (3.5%, 22.8% and 17.2% of total fatty acids, respectively) and a good source of essential amino acids. Despite equal growth, dietary apparent digestibility coefficient of protein was 3% and of fat 0.7% lower in the tunicate meal diet as compared to the control, which may have been due to the higher ash levels (ca. 25% including salt) in the test material (Kousoulaki et al., 2020).

The technical properties of fishmeal (FM) and plant proteins as soy protein concentrate (SPC) and vital wheat gluten (WG) have been reported in several studies and compared to FM and WG, the SPC has higher dough viscosity and requires higher temperature and mechanical shearing for successful plasticisation in the extrusion process (Draganovic et al., 2011, 2013; Sørensen et al., 2009; Samuelsen et al., 2013, 2014, 2018). Such published information is still lacking for the new and sustainable protein source, tunicate meal, and the aim for this research was therefore to study the technical properties of tunicate meal, by quantifying (1) the effect of the replacement of FM or SPC by tunicate meal on dough viscosity, extrudate expansion, microstructure, physical pellet quality, oil adsorption and leakage; (2) in combination with FM and SPC, assess the maximum tunicate inclusion level possible to use without compromising physical pellet quality.

## 2. Materials and methods

### 2.1. Ingredients and feed mix composition

Seven experimental feed mixes were formulated. Except of the new protein source, tunicate meal, all other ingredients are commonly used in the Norwegian salmon feed industry (Aas et al., 2019; Tables 1 and 2). The feed mixes were standardised to an equal moisture level by addition of water (81 g/kg wet basis). Water was sprayed homogeneously into the feed mixes at least 24 h before processing to secure even partitioning and adsorption into the feed matrix.

**Table 1**
Proximate chemical composition of the feed mix ingredients (g/kg).

| | Tunicate[b] | FM[c] | SPC[d] | WG[e] | CGM[f] | Faba[g] | Wheat[h] |
|---|---|---|---|---|---|---|---|
| DM | 969 | 922 | 929 | 956 | 921 | 869 | 880 |
| In DM | | | | | | | |
| Crude protein | 368 | 783 | 675 | 857 | 682 | 323 | 156 |
| Lipid | 35 | 112 | 10 | 43 | 43 | 23 | 24 |
| Total ash | 355 | 130 | 69 | 7 | 11 | 33 | 102 |
| Carbohydrate[a] | 241 | – | 247 | 93 | 264 | 620 | 718 |

CGM, corn gluten meal; DM, dry matter; Faba, horse beans; FM, fishmeal; SPC, soy protein concentrate; WG, vital wheat gluten; Wheat, whole wheat flour.

[a] Estimated based on the crude protein, lipid and total ash level.

[b] *Ciona intestinalis*, produced by Marine Feed Sweden AB (Stenungsund, Sweden).

[c] Purchased from Pelagia (Egersund, Norway).

[d] Purchased from Agro Korn AS (Videbæk, Denmark).

[e] Purchased from Tereos Syral (Aals, Belgium).

[f] Purchased from Roquette (Lestrem, France)

[g] Purchased from Soufflet (Nogent-sur-Seine, France)

[h] Bakery quality (falling number >200 s), purchased from Norgesmøllene AS (Vaksdal, Norway).

**Table 2**
Formulation and proximate chemical composition of the experimental feed mixes fed to the extruder system (g/kg DM).[a]

| | EX1 | EX2 | EX3 | EX4 | EX5 | EX6 | EX7 |
|---|---|---|---|---|---|---|---|
| Formulation | | | | | | | |
| Tunicate | 345 | 0 | 0 | 172 | 172 | 0 | 115 |
| FM | 0 | 345 | 0 | 172 | 0 | 172 | 115 |
| SPC | 0 | 0 | 345 | 0 | 172 | 172 | 115 |
| WG | 215 | 215 | 215 | 215 | 215 | 215 | 215 |
| Wheat | 138 | 138 | 138 | 138 | 138 | 138 | 138 |
| CGM | 69 | 69 | 69 | 69 | 69 | 69 | 69 |
| Faba | 110 | 110 | 110 | 110 | 110 | 110 | 110 |
| Rapeseed oil | 56 | 56 | 56 | 56 | 56 | 56 | 56 |
| Mineral mixture | 7 | 7 | 7 | 7 | 7 | 7 | 7 |
| Vitamin mixture | 7 | 7 | 7 | 7 | 7 | 7 | 7 |
| Monosodium phosphate | 34 | 34 | 34 | 34 | 34 | 34 | 34 |
| DL-Methionine | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| L-Histidine | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| L-Lysine | 14 | 14 | 14 | 14 | 14 | 14 | 14 |
| Chemical composition[b] | | | | | | | |
| Crude Protein | 433 | 575 | 538 | 504 | 485 | 557 | 515 |
| Lipid | 85 | 112 | 77 | 99 | 81 | 94 | 91 |
| Total Ash | 186 | 109 | 88 | 148 | 137 | 98 | 128 |
| Carbohydrate | 298 | 214 | 299 | 256 | 298 | 257 | 270 |

CGM, corn gluten meal; DM, dry matter; EX, experimental feed mix; Faba, horse beans; FM, fishmeal; SPC, soy protein concentrate; WG, vital wheat gluten; Wheat, whole wheat flour.
[a] Proximate chemical composition calculated for diets at a lipid level of 338 g/kg DM and a starch level of 100 g/kg DM: Crude Protein, 374 ± 34.7 g/kg; Total ash, 98 ± 20.6 g/kg
[b] Calculated based on raw material analyses (Table 1).

### 2.2. Extrusion and drying conditions

The feed mixes, calibrated to 150 kg/h, were conditioned in an atmospheric double differential preconditioner (Wenger Manufacturing Inc., Sabetha, KS) prior to extrusion on a TX-52 co-rotating, fully intermeshing twin-screw extruder (Wenger). A standard shaft speed (220 rpm) and addition of steam (12.6 kg/h) and water (6.0 kg/h) in the preconditioner were used for all trials, giving a preconditioner outlet temperature of 86 ± 1.8 °C and a moisture content of 166 ± 3.3 g/kg (wet basis). Extruder steam and water addition were 3.0 kg/h and 12.0 kg/h, respectively giving a total moisture content 235 ± 2.8 g/kg (wet basis) behind the extruder die. The screw configuration was the same as described in Samuelsen and Oterhals (2016) and the screws were run at a constant speed of 400 rpm. The extruder outlet was restricted by three circular 6.1 mm dies. The extrudates were cut at the die surface with a constant knife speed at 950 rpm (2 knifes). Specific mechanical energy (SME) was calculated based on the measured extruder torque (kW) and wet flow rate (kg/h) as described in Samuelsen et al. (2018).

The feed dough temperature ($T_{die}$) and pressure were measured in the product stream behind the extruder die. Apparent shear rate, shear stress and viscosity of the feed dough in the die ($V_{die}$) were calculated by use of the die diameter (6.1 mm) and length (6.3 mm) and the measured pressure and the volume flow rate as described by Samuelsen et al. (2018). Sampling was conducted after achievement of steady state conditions in the preconditioner and extruder. During a period of 5 min, extrudates were pre-dried in a in a hot air dual layer carousel dryer (Model 200.2, Paul Klöckner GmbH, Nistertal, Germany) at constant air temperature (70 °C) to stabilise the pellet structure. Final drying was performed in a laboratory hot air dryer (Dörrex, A. & J. Stöckli AG, Netstal, Switzerland) at 70 ºC to a moisture content of 70 ± 5.2 g/kg (wet basis). Duplicate runs were performed for four formulations (Table 3) as follows: 5

**Table 3**
Pseudo and actual units for the mixture design variables, tunicate meal, fishmeal (FM) and soy protein concentrate (SPC).

| Feed mix | ENo | Pseudo units | | | Tunicate (g/kg DM) | FM (g/kg DM) | SPC (g/kg DM) |
|---|---|---|---|---|---|---|---|
| EX1–1 | 5a | 1 | 0 | 0 | 345 | 0 | 0 |
| EX1–2 | 5b | 1 | 0 | 0 | 345 | 0 | 0 |
| EX2–1 | 4a | 0 | 1 | 0 | 0 | 345 | 0 |
| EX2–2 | 4b | 0 | 1 | 0 | 0 | 345 | 0 |
| EX3–1 | 2a | 0 | 0 | 1 | 0 | 0 | 345 |
| EX3–2 | 2b | 0 | 0 | 1 | 0 | 0 | 345 |
| EX4–1 | 3a | 0.5 | 0.5 | 0 | 172 | 172 | 0 |
| EX4–2 | 3b | 0.5 | 0.5 | 0 | 172 | 172 | 0 |
| EX5 | 7 | 0.5 | 0 | 0.5 | 172 | 0 | 172 |
| EX6 | 6 | 0 | 0.5 | 0.5 | 0 | 172 | 172 |
| EX7 | 1 | 0.33 | 0.33 | 0.33 | 115 | 115 | 155 |

DM, dry matter; ENo, experiment number order; EX, experimental feed mix.

**Table 4**
Extruder, pellet and microstructure responses.

| | Extruder | | | Pellet | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Feed mix | SME (Wh/kg) | $T_{die}$ (°C) | $V_{die}$ (Pa s) | Hardness (N) | Dur. (%) | SEI | Length (mm) | Bulk density (g/L) | Max fat[a] (g/kg) | Fat leakage (g/kg) | WSI (%) | Total porosity (%) | Open porosity (%) | Closed porosity (%) | MCS (μm) |
| EX1–1 | 38.4 | 108 | 253 | 114.1 | 73.5 | 2.36 | 12.84 | 346 | 459 | 142 | 35.0 | 56.9 | 56.8 | 0.2 | 422.5 |
| EX1–2 | 38.6 | 112 | 279 | 111.5 | 74.8 | 2.27 | 12.64 | 351 | 451 | 145 | 32.6 | 54.1 | 54.0 | 0.3 | 390.9 |
| EX2–1 | 30.8 | 96 | 416 | 111.3 | 87.1 | 2.18 | 10.03 | 520 | 249 | 87.9 | 74.0 | 30.2 | 29.8 | 0.6 | 136.7 |
| EX2–2 | 31.4 | 100 | 445 | 108.2 | 87.5 | 2.08 | 9.68 | 524 | 239 | 77.8 | 80.4 | 29.9 | 29.4 | 0.7 | 125.2 |
| EX3–1 | 42.4 | 108 | 528 | 96.3 | 78.9 | 2.00 | 10.69 | 484 | 288 | 89.3 | 68.6 | 29.7 | 29.3 | 0.6 | 137.8 |
| EX3–2 | 42.5 | 110 | 534 | 96.0 | 79.7 | 2.00 | 10.66 | 486 | 281 | 85.5 | 64.8 | 30.2 | 29.8 | 0.6 | 133.5 |
| EX4–1 | 31.4 | 105 | 380 | 103.6 | 87.0 | 2.45 | 10.54 | 425 | 362 | 98.8 | 58.5 | 45.2 | 45.0 | 0.4 | 371.0 |
| EX4–2 | 31.9 | 103 | 347 | 113.3 | 88.8 | 2.46 | 10.60 | 426 | 360 | 109 | 63.8 | 47.8 | 47.6 | 0.4 | 349.3 |
| EX5 | 38.1 | 106 | 397 | 95.9 | 77.2 | 2.22 | 11.29 | 412 | 374 | 104 | 56.7 | 44.0 | 43.7 | 0.5 | 278.6 |
| EX6 | 35.9 | 112 | 458 | 115.5 | 82.3 | 1.94 | 10.54 | 494 | 263 | 83.4 | 76.7 | 28.2 | 27.7 | 0.7 | 113.3 |
| EX7 | 32.7 | 102 | 384 | 91.3 | 81.5 | 1.98 | 10.01 | 476 | 296 | 90.4 | 71.3 | 34.4 | 34.0 | 0.6 | 159.5 |

Dur., Holmen durability; Max fat, oil adsorption capacity; MCS, mean cell size; SEI, sectional expansion index; SME, specific mechanical energy; $T_{die}$, temperature behind extruder die; $V_{die}$, apparent viscosity in the extruder die; WSI, water stability index.
[a] g/kg of total oil adsorbed.

min sampling, 5 min run, 5 min sampling.

### 2.3. Chemical analyses

Dry matter (DM) was measured gravimetrically after drying at 103 ± 1 °C (ISO 6496, 1999). Crude protein (N x 6.25) was analysed by the Kjeldahl method (ISO 5983-2, 2009). Fat content was determined based on petroleum ether Soxhlet extraction before and after heating with hydrochloric acid (COMMISSION REGULATION (EC) No 152) and ash was determined by combustion of organic matter at 550 °C and gravimetric measurement of the residue remaining (ISO 5984, 2002). Total starch and degree of starch gelatinisation were measured utilising a modification of the glucoamylase methodology described by Chiang and Johnson (1977) and Samuelsen and Oterhals (2016). All chemical measurements were based on averages of duplicate analyses.

### 2.4. Analysis of extruded pellet properties

Hardness (peak breaking force) was measured on uncoated laying pellets by use of a texture analyzer (TA.XTplusC, Stable Micro Systems Ltd, Surrey, UK). The speed of the load arm was set to 1 mm/s and the penetration depth to 4.0 mm. The load arm was equipped with a cylindrical flat-ended probe (75 mm diameter). Pellets were treated individually and reported values based on the average of 20 analyses. Durability was measured on uncoated pellets using a Holmen pellet tester (Holmen Feed Technology, Berkshire, UK), where a sieved (8 mm screen) 100 g pellet sample was conveyed around in a closed circuit by a high velocity air stream as described in Samuelsen et al. (2013). Durability were based on the average of duplicate measurements. Diameter and length were measured with an electronic calliper and based on averages of 20 pellets. Sectional expansion index (SEI) was calculated by dividing the square of the pellet diameter by the square of the die diameter. Bulk density was measured by loosely pouring the dry uncoated pellets from a funnel into a 1000 ml measuring cylinder. Reported values were based on the average of triplicates. Oil adsorption capacity (Max fat) was investigated using a laboratory vacuum coater (Type PG-10VC LAB, DINNISSEN, Sevenum, Netherlands). A 500 g uncoated pellet sample and 1000 g pre-heated rapeseed oil (60 °C) were added into the vacuum coater and the air withdrawn. The air was slowly released back to the coater during moderate mixing (1 min) to obtain a maximal oil filling of the pellet pores. Surplus oil was rinsed off and the coated pellet was stabilised for 60 min at 23 °C before weighing. Reported values were based on the average of duplicate measurements. Fat leakage was measured on the samples from the previous test. The sample was transferred into a closed plastic box with blotting paper and the pellet weight recorded. After incubation at 40 °C for 24 h in a heating cabinet the sample weight was registered, and fat leakage calculated. Reported oil adsorption capacity and fat leakage were based on the average of duplicate measurements. Pellet water stability index (WSI) was determined utilising a slightly modified methodology described by Baeverfjord et al. (2006). Triplicate samples of each diet (20 g each) were placed in custom made steel-mesh buckets placed inside 1000 ml glass beakers filled with 500 ml distilled water. The beakers were incubated in a thermostat-controlled water bath (23ºC) and shaken (160/min) for 120 min, and the remaining amount of DM was determined.

### 2.5. Pellet microstructure

Micro-CT scanning (Landis and Keane, 2010) was preformed using a Skyscan 1275 X-ray microtomograph (Bruker micro-CT, Kontich, Belgium). The pixel size was set to 12 μm, voltage to 30 kV and current to 212 μA. The scans were reconstructed using NRecon (v. 1.7.3.1, Bruker micro-CT, Kontich, Belgium). The porosity calculations were performed using CTan (v. 1.17.7.2 +, Bruker micro-CT, Kontich, Belgium). The threshold was set using the Automatic Otsu function. Percent porosity (total, open, closed), mean cell size (MCS) and cell size and cell wall thickness were calculated as described in Ahmad et al. (2019).

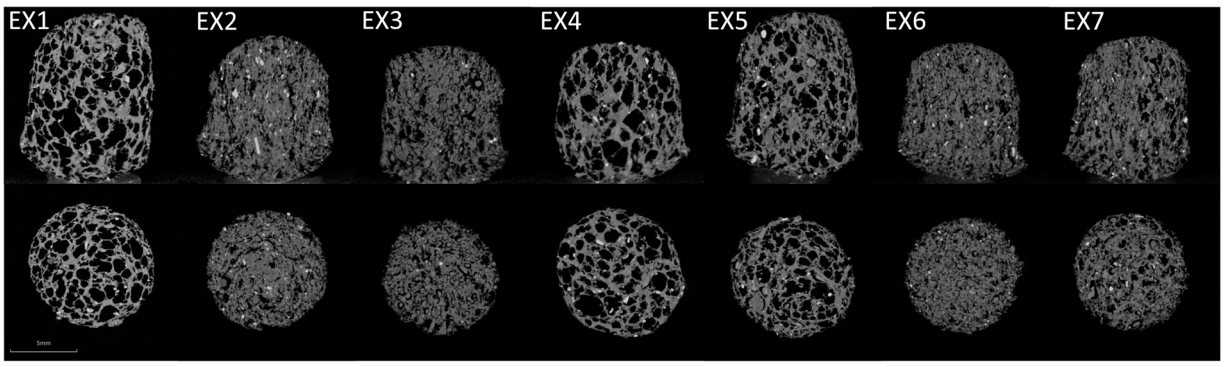


**Fig. 1.** X-ray microtomography images in longitudinal (top) and radial (bottom) views for the pellet samples. Black correspond to air, grey to solid structure, and white to minerals and fish bone fragments. Detailed composition data are given in Table 2.

### 2.6. Experimental design and statistical analyses

The experiment was based on a 3-component simplex-centroid mixture design by varying the content of tunicate, FM, and SPC in the feed mixes (Table 2). The design, fitted to Scheffe special cubic polynomial model, represents a triangle with a total of 7 experimental settings including three vertex points (pure blends), three edge points (binary blends), and a centroid point (Cornell, 2002). The vertex points and one edge points were replicated (Table 3). A fraction of 345 g/kg DM of the feed mix was varied giving a range of 0–345 g/kg DM for the three ingredients. The rest of the feed mix was held constant (Table 2). The experiments were run in random order (Table 3). The extruder, pellet and microstructure responses are given in Table 4. Design-Expert v10 (Stat-Ease, Inc. Minneapolis, USA) was used for mixture design modelling and numerical optimisation based on pseudo scaled components. Principal component analysis (PCA; Martens and Martens, 2001) was performed using Unscrambler 10.5 (Camo, Oslo, Norway) on mean centred and standardised variables to assesses the data structure of the mixture variables and responses. Basic statistics were carried out using STATISTICA v13.5 (StatSoft, Inc. Tulsa, USA).

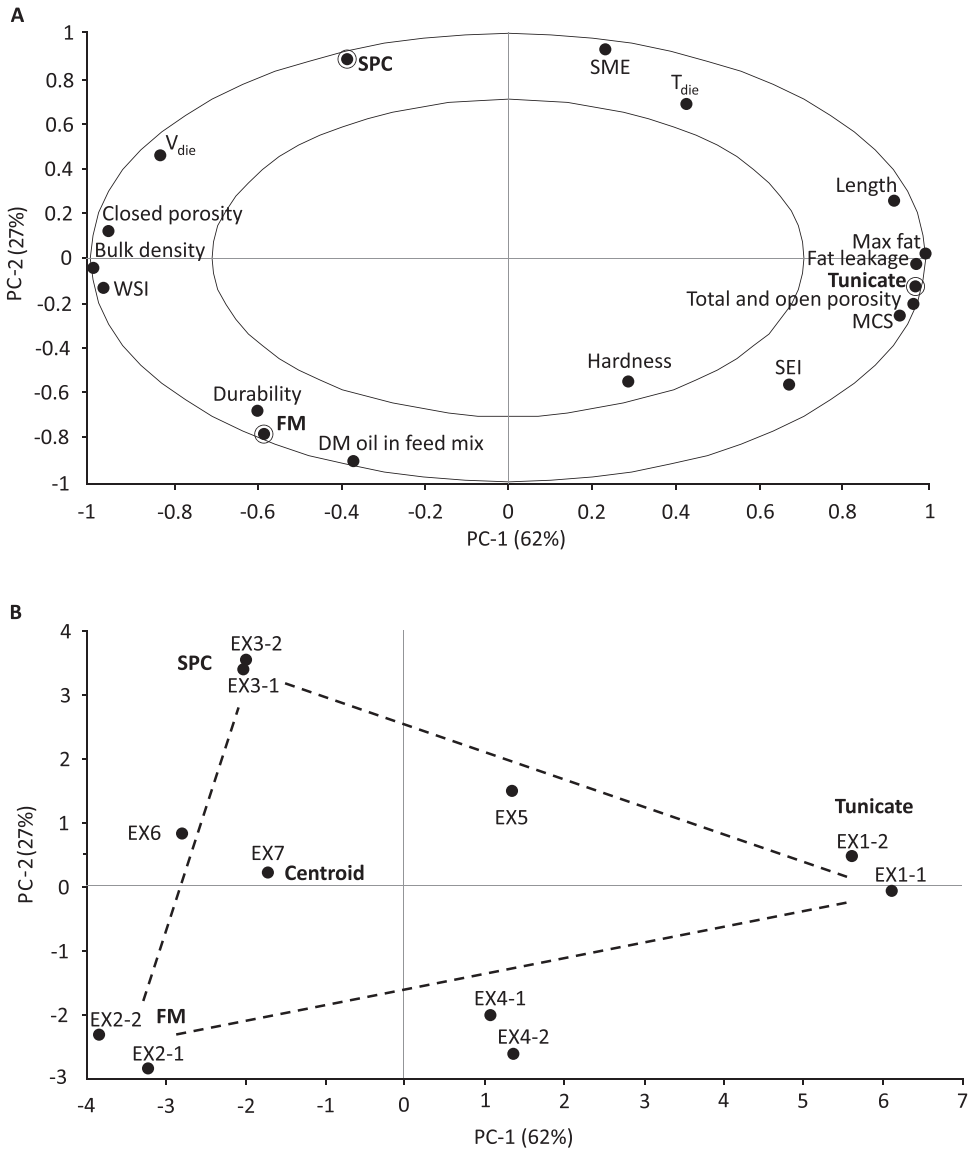


**Fig. 2.** Principal component analysis (PCA). A) Correlation loading plot based on the mixture design and response variables. The two ellipses represent 50% and 100% of explained variance. B) Score plot showing similarities in responses based on the applied mixture design variables. Abbreviations are explained in Tables 3 and 4.

## 3. Results

### 3.1. Data overview

The experimental conditions resulted in a large span in the measured extruder and pellet responses and different microstructure (Table 4, Fig. 1). The PCA loading plot visualise the relationships between the design variables and responses (Fig. 2A) and with principal component (PC) 1 explaining 62% and PC2 27% of the variance. Effects of tunicate were mainly explained by PC1, and effects of FM and SPC by PC2. The corresponding score plot (Fig. 2B) shows similarities among the samples and reflects combinations of conditions in the experimental design that give comparable responses. The four replicate points confirms good experimental reproducibility. The centroid point sample (EX 7) showed similarities to the pure FM (EX2) and SPC (EX3) blends and the binary blend of FM and SPC (EX6).

Increase in the extruder response SME was mainly explained by the reduced lipid content in the feed mix ($R^2 = 0.830$) due to the replacement of FM with tunicate or SPC (Table 4, Fig. 2A). Increased SME have resulted in higher $T_{die}$ ($R^2 = 0.560$). $V_{die}$ was impacted by the tunicate level in the feed mix ($R^2 = 0.835$). All samples were properly cooked with high degree of starch gelatinisation i.e. 99.2 ± 2.1% of a total and equal starch content of 168 ± 3.0 g/kg. The pellet response, hardness was explained by PC3 (5%; not shown) and was negatively associated with SPC. The highest durability was found for the pure FM blend. Replacement of FM or SPC by tunicate gave the largest change in pellet expansion (SEI, length, bulk density, max fat) and microstructure parameters, as increased pore volume (open and total porosity), larger average pore sizes (MCS) and a broader cell size distribution (Figs. 3 and 4A). The highly correlated total and open porosity ($R^2 = 1$) were on average 39.1 ± 10.7% and 38.8 ± 10.9% of the total pore volume, respectively.

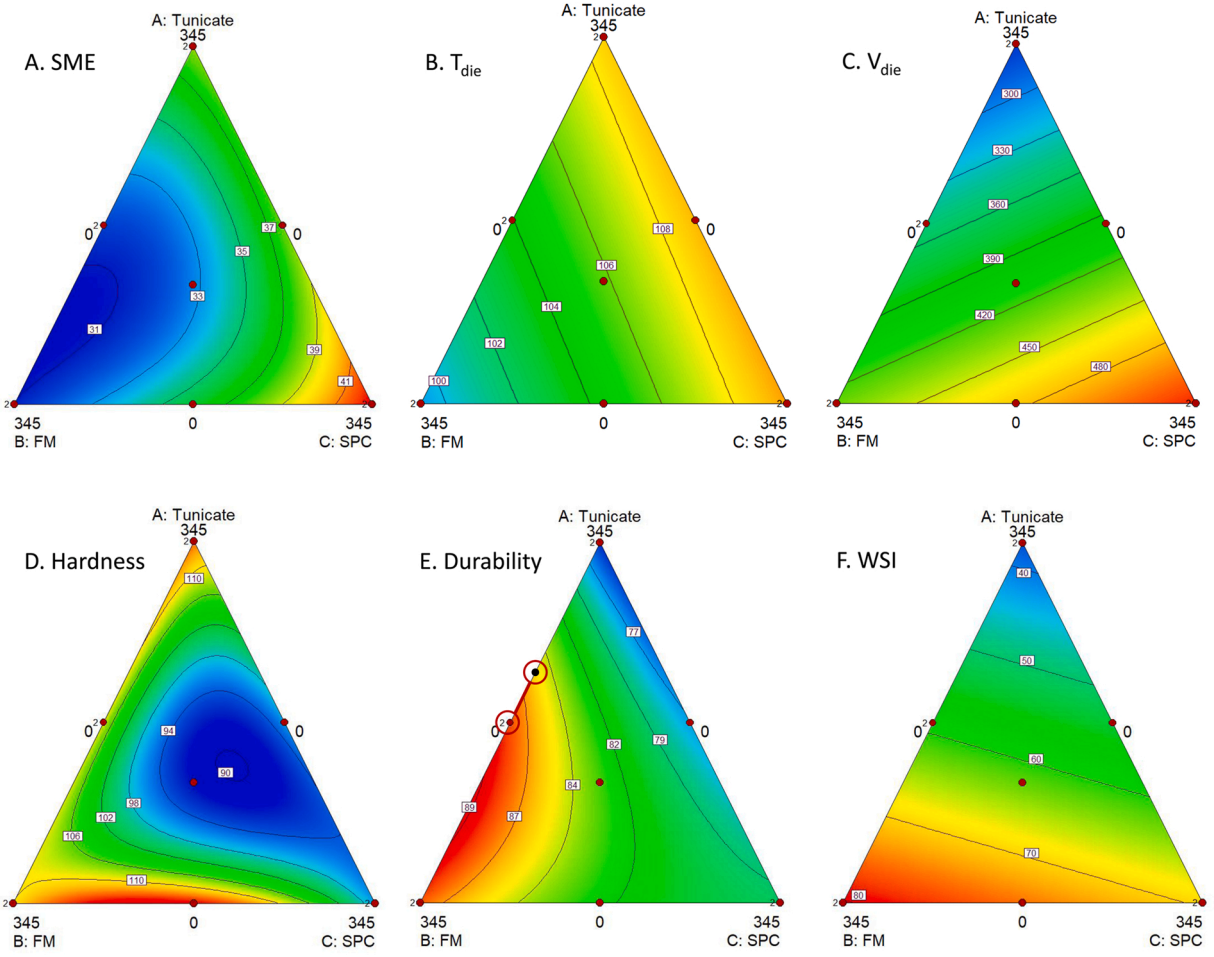


**Fig. 3.** Contour plots for the extruder, pellet and microstructure responses. Actual inclusion levels (g/kg DM) for the three components are given for the pure (vertex) and binary (edge) points. The contours are flagged with the actual unit of the respective response. Solid red circles represent the design points. A) Specific mechanical energy (SME; Wh/kg), B) Temperature behind the die ($T_{die}$; °C), C) Apparent dough viscosity in the extruder die ($V_{die}$; Pa s), D) Hardness (N), E) Durability (%). The circles defines the local optimums from the numerical optimisation, F) Water stability index (WSI; %), G) Bulk density (g/L), H) Oil adsorption capacity (Max fat; g/kg), I) Fat leakage (g/kg), J) Open porosity (%), K) Closed porosity (%), L) Mean cell size (MCS; μm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

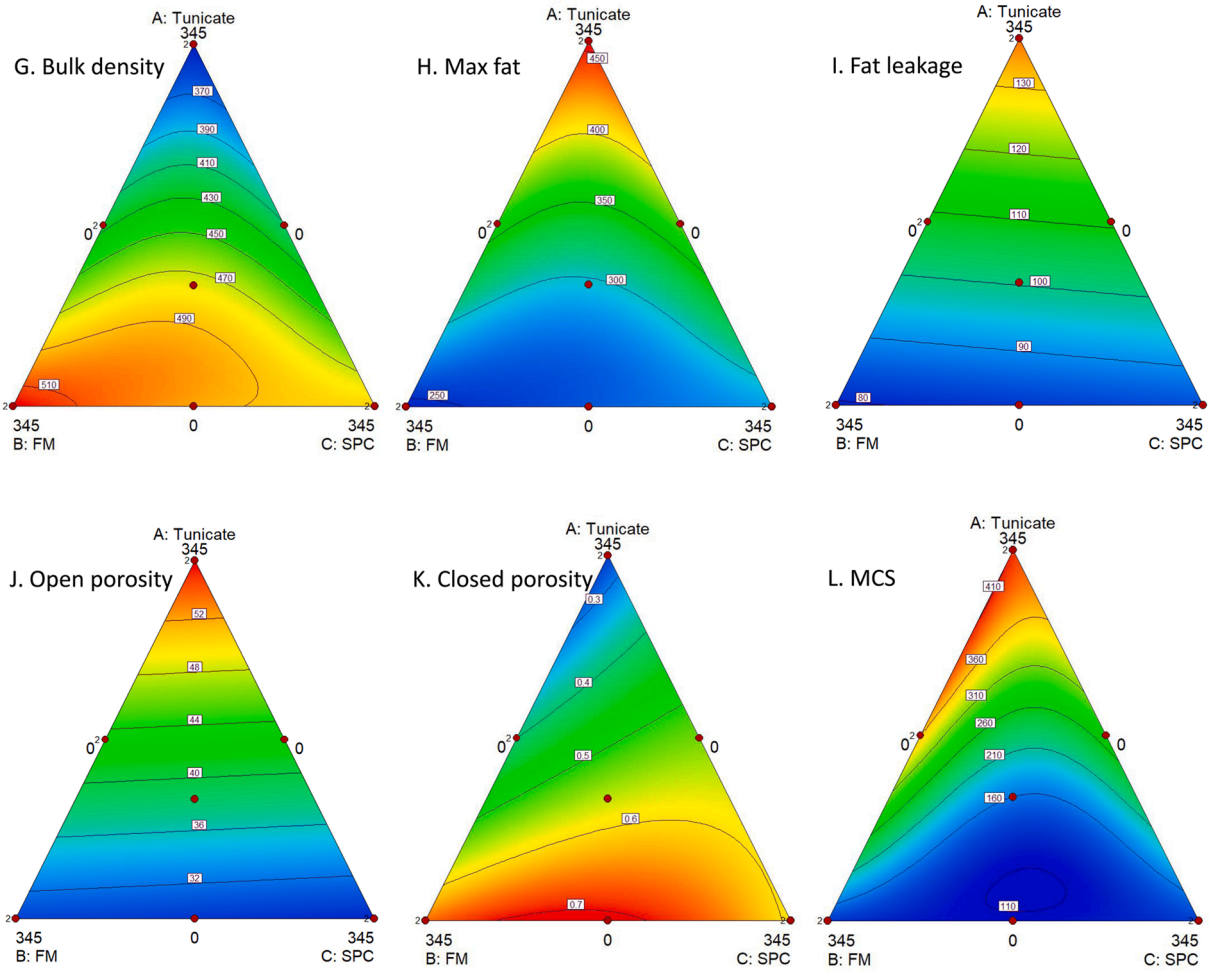


**Fig. 3.** (*continued*).

Closed porosity amounted to only 0.51 ± 0.16% of the total volume, i.e. most of the volume was open and in line with other studies (Bhatnagar and Hanna, 1997; Ahmad et al., 2019). Bulk density and max fat showed a strong negative, linear correlation ($R^2 = 0.997$) and both correlated to open porosity ($R^2 = 0.945$ and 0.928, respectively). The highest max fat value was found for the pure tunicate blend. Fat leakage were positively correlated to max fat ($R^2 = 0.914$). All samples leaked out approximately same percent of adsorbed fat (31 ± 2.2%), i.e. there is a linear correlation between max fat and the retained oil after fat leakage test ($R^2 = 0.986$). The tunicate pellet showed reduced WSI. WSI was positively correlated to bulk density ($R^2 = 0.921$) and negatively correlated to open pores ($R^2 = 0.812$).

### 3.2. Mixture models

Based on the 3-component mixture design (Table 3), models with high explained variance were established for all the responses ($R^2 = 0.916–0.999$, P = 0.0270 to < 0.0001; Table 5) except for $T_{die}$ where a weaker model was found ($R^2 = 0.574$, P = 0.0300). No outliers were detected. All linear and quadratic models showed insignificant lack of fit. For the special cubic models, the number of runs (degrees of freedom) were used for modelling and lack of fit could not be estimated.

The response SME was fitted to special cubic model and $T_{die}$ to a linear model (Table 5) and with the highest values found on the edge of the binary tunicate and SPC blends and lowest value on the vertex of the pure FM blend (Table 5, Fig. 3A, B). $V_{die}$ were also fitted to a linear model. The highest value was found on the vertex of the pure SPC blend and the lowest value on the vertex of the pure tunicate blend (Fig. 3C).

Hardness was fitted to a special cubic model and durability to a quadratic model (Table 5). Hardness was lowest for blends high in SPC and in the interior of the triangle (Fig. 3D). Durability was highest for the pure FM and binary tunicate and FM blends and with the lowest value found for the pure tunicate blend (Fig. 3E).

The expansion parameters, SEI, length, bulk density and max fat were all fitted to special cubic models, and fat leakage to a linear model. All models were mainly affected by the tunicate level in the three-component blend (Table 5, Fig. 3G–I). The same pattern was

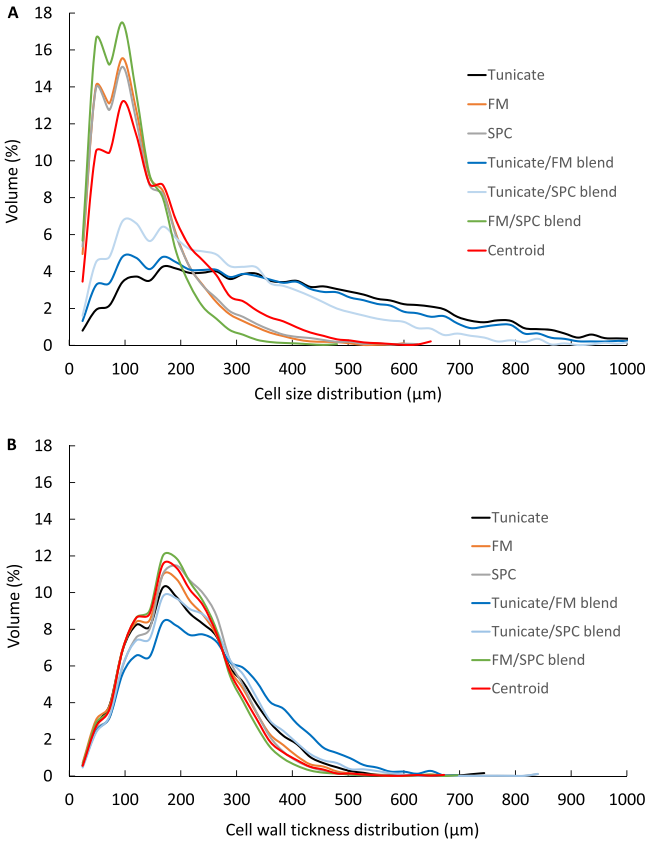


**Fig. 4.** A Cell size-volumetric distribution for the pellet samples. B Cell wall thickness-volumetric distribution for the pellet samples.

**Table 5**
Mixture models.

| Variables | Pseudo Component model | P - value | Lack of fit (P) | $R^2$ |
|---|---|---|---|---|
| SME[a] | 38.5 A+ 31.1 B + 42.5 C - 12.5 AB - 9.4 AC - 3.4 BC - 48.4 ABC | < 0.0001 | na | 0.998 |
| $T_{die}$[b] | 108.6 A + 99.3 B + 109.5 C | 0.0300 | 0.088 | 0.574 |
| $V_{die}$[b] | 269.4 A + 429.1 B + 522.8 C | < 0.0001 | 0.479 | 0.965 |
| Hardness[a] | 112.8 A + 109.7 B + 96.1 C - 11.22 AB - 34.4 AC + 50.3 BC - 416.4 ABC | 0.027 | na | 0.929 |
| Durability[c] | 74.2 A + 87.4 B + 79.3 C + 27.5 AB - 0.4 AC - 6.1 BC | 0.0002 | 0.191 | 0.984 |
| SEI[a] | 2.31 A + 2.13 B + 2.00 C + 0.94 AB + 0.25 AC - 0.51 BC - 6.60 ABC | 0.0040 | na | 0.974 |
| Length[a] | 12.74 A + 9.86 B + 10.68 C - 2.92 AB - 1.67 AC + 1.07 BC - 18.70 ABC | 0.0004 | na | 0.992 |
| Bulk density[a] | 348.2 A + 521.8 B + 484.9 C - 37.6 AB - 18.6 AC - 35.5 BC + 929.7 ABC | < 0.0001 | na | 0.999 |
| Max fat[a] | 454.8 A + 244.1 B + 284.3 C + 45.6 AB + 17.4 AC - 3.3 BC - 1038.9 ABC | < 0.0001 | na | 0.998 |
| Fat leakage[b] | 137.6 A + 79.3 B + 84.3 C | < 0.0001 | 0.181 | 0.919 |
| WSI[b] | 37.3 A + 80.3 B + 69.5 C | < 0.0001 | 0.117 | 0.916 |
| Total porosity[b] | 56.6 A + 30.4 B + 29.1 C | < 0.0001 | 0.057 | 0.955 |
| Open porosity[b] | 56.5 A + 30.0 B + 28.7 C | < 0.0001 | 0.056 | 0.955 |
| Closed porosity[c] | 0.26 A + 0.66 B + 0.59 C – 0.23 AB + 0.44 AC + 0.34 BC | 0.0002 | 0.845 | 0.983 |
| MCS[a] | 406.7 A + 131.0 B + 135.7 C + 365.2 AB + 29.5 AC − 79.9 BC - 2697.5 ABC | 0.0002 | na | 0.995 |

A = Tunicate meal, B = fishmeal, C = soy protein concentrate.
na, not applicable because the degrees of freedom are used for modelling.
Abbreviations explained in Table 4.
[a] Special cubic model.
[b] Linear model.
[c] Quadratic.

also found for the linear WSI model (Fig. 3F) and for the microstructure parameters total and open pores (linear) closed pores (quadratic) and MCS (special cubic; Table 5, Fig. 3J–L).

### 3.3. Numerical optimisation

A numerical response optimisation was performed on the data set by maximising tunicate level in the feed mix, hardness, durability, open pores, max fat and WSI. The maximum tunicate inclusion level possible to use without compromising physical pellet quality can be determined by using these criteria. The suggested boundary levels and best local optimums were found on the edge of the of the tunicate and FM binary blends, ranging from EX4 to the blend corresponding to 220 g/kg DM tunicate, 125 g/kg DM FM and 0 SPC g/kg DM (Tables 2 and 3, Fig. 3E).

## 4. Discussion

This study reports the effect of the replacement of FM or SPC by tunicate meal on the extrusion process, physical pellet quality and microstructure, and document large differences in the technical quality of the studied ingredients.

### 4.1. Effect on SME, $T_{die}$, and $V_{die}$

SME is a measure of the sum of the total mechanical energy dissipated in the extruder screw and are converted in to heat (Godavarti and Karwe, 1997; Della Valle et al., 1989). This can be seen by the positive correlation of SME with $T_{die}$ in this study (Fig. 2A). The increase in the responses SME and $T_{die}$ were mainly explained by reduced lipid level in the feed mix, given by the replacement of FM by tunicate or SPC (Table 2, Fig. 2A). The effect can be attributed to the lubrication effect of the lipids. Samuelsen et al. (2018) reported that an increase in SME and $T_{die}$ linearly reduced the apparent dough viscosity in the extruder die ($V_{die}$). This cannot be deducted from this study were $V_{die}$ reduction mainly was an effect of increased tunicate level in the feed mix and with the lowest value found for the pure tunicate blend (Fig. 3C). The highest $V_{die}$ was found for the pure SPC blend. An interesting observation is a nearly equal $T_{die}$ for the pure tunicate and SPC blends and for all binary combinations of the two ingredients (Fig. 3B). Due to the standardised extrusion conditions, they have also the same moisture content, meaning that at an equal temperature and moisture level the pure tunicate blend has lower apparent dough viscosity than the pure SPC blend. The observed lower viscosity for tunicate compared to SPC may be due to a lower glass transition temperature ($T_g$), the mobilisation of more protein groups above $T_g$ and increased effect of temperature on viscosity reduction in the rubbery state (Bengoechea et al., 2007; Oterhals et al., 2019). This can be verified by use of capillary rheometry but is outside the scope of this study.

### 4.2. Effect on pellet hardness and durability

The moisture content in the preconditioner and extruder were in total 235 ± 2.8 g/kg and were chosen from a test trail in order to produce satisfying pellet quality in all the design points. In the extrusion process each powdery protein ingredient can be seen as a separate phase requiring different moisture content and temperature conditions for plasticisation to a dough in the extrusion process. Achieving these conditions for all the ingredients in the feed mix are crucial for obtaining new intermolecular binding networks and acceptable physical product quality. In Oterhals et al. (2019) a critical moisture level for plasticisation of SPC was found in the range of 233–306 g/kg. In the same study a critical moisture level of 138 g/kg was determined for a FM. The used moisture content in this study (235 g/kg) is in the low end found for SPC plasticisation. Pellet from the pure SPC blend had significantly lower hardness and durability compared to the pure FM blend (Table 4, Fig. 3D, E) and may be a result of incomplete plasticisation of the SPC blend at this low moisture content. This will increase the level of solid particles in the extrudate resulting in poor pellet hardness and durability (Areas, 1992; Mitchell and Arêas, 1992). Using a higher moisture content in the process would probably increase plasticisation of the SPC blend and improve physical pellet quality. Low durability for the pellet high in tunicate meal (Fig. 3E) may be explained by another mechanism. These are the pellets with lowest bulk density and highest expansion (SEI, length; Figs. 1, 2A and 3G), indicating a negative effect of increased expansion on durability. This agrees with other studies performed on extruded food foams and fish feed (Hayter et al., 1986; Sørensen, 2012). There was however no correlation between hardness and durability in this study and with all the samples showing high hardness (Table 4, Fig. 2A).

### 4.3. Effect on expansion parameters and porosity

In the extruder die, because of elevated temperature and pressure, moisture is in its liquid state in the feed dough. At die exit, the dough enters the atmosphere and steam flashes off. This process creates bubbles by nucleation of steam in the dough. Because of the high steam pressure bubbles will then rupture through the cell walls and an open porous structure in the extrudate is formed (Fan et al., 1994; Moraru and Kokini, 2003). Due to a sudden decrease in extrudate moisture and temperature during the steam flash of, viscosity reduction and fixation of the extrudate structure will occur. Increased expansion and porosity can be achieved with higher steam flashing rate, i.e. increase in temperature behind the extruder die and/or by a reduction in dough viscosity (Della Valle et al., 1997; Fan et al., 1994; Moraru and Kokini, 2003; Ahmad et al., 2019). In this study, these mechanisms were clearly seen. The binary blends of tunicate meal and SPC have equal steam flashing rate (similar $T_{die}$; Fig. 3B) but with a significant reduction in dough viscosity at increased tunicate level ($V_{die}$; Fig. 3C). The observed viscosity reduction have resulted in increased pellet expansion (Fig. 1, EX1 and 3)

and open porosity (Fig. 3J), larger pores (MCS; Fig. 3L) and a broader cell size distribution (Fig. 4A). By replacement of FM by tunicate meal, increased pellet expansion and porosity were also observed (Fig. 1, EX1 and 2). This is a combined effect of increased steam flash flashing rate (higher $T_{die}$; Fig. 3B) and reduced dough viscosity ($V_{die}$; Fig. 3C). Replacement of FM by SPC had low impact on pellet expansion (Fig. 1, EX2 and 3). The increase in steam flashing rate for the SPC blend (higher $T_{die}$; Fig. 3B) have been counteracted by a reduction in dough viscosity ($V_{die}$; Fig. 3C).

### 4.4. *Effect of microstructure on max fat, leakage, and water stability*

Bulk density and max fat showed a strong negative, linear correlation, also observed in other studies (Samuelsen and Oterhals, 2016; Samuelsen et al., 2018). Both were correlated to open porosity and the pellet samples that adsorbed the highest amount of fat (tunicate blends) also had highest number of open pores (Figs. 1 and 3H, J). These pellet samples also showed highest fat leakage. However, all samples leaked out approximately same percent of the adsorbed fat, meaning that the tunicate blends still have the highest amount of retained fat after the test (Fig. 5). Due to the higher MSC and a broader cell size distribution for the tunicate blends (Figs. 3L and 4A), it was expected a higher percent of fat leakage compared to the SPC and FM blends. The high retained fat content for the tunicate blends may be explained by layers of oil on the surface of the larger pores not leaked out during testing (Dethlefsen, 2017).

Water stability may impact the biological response of the fish (Hilton et al., 1981; Baeverfjord et al., 2006; Sørensen, 2012). The WSI (Fig. 3F) was positively correlated to bulk density and was reduced with a higher number of open pores (Fig. 2A). The lowest WSI was found for pellet high in tunicates (Fig. 3F). WSI was performed on uncoated pellet. The cell wall thickness distribution (Fig. 4B) showed low variation with only a slightly broader distribution for the tunicate blends. The increased pellet degradation speed for the tunicate blends (reduced WSI) may therefore be a result of faster water penetration and higher dissolution rate of the cell walls due to the higher pore volume (Fig. 3J) and larger cell sizes (Fig. 3L and 4A). However, the degree of cell-wall homogeneity and type of bonds may also influence WSI.

This study documents that microstructure parameter measured by X-ray microtomography can give valuable information about pellet properties and in line with other studies (Bhatnagar and Hanna, 1997; Agbisit et al., 2007; Robin et al., 2010; Ahmad et al., 2019).

### 4.5. *Numerical optimisation*

This study was for technical purposes only and the feed mixes nutritional quality were not considered. Based on the chosen standardised extrusion setup and conditions maximum tunicate inclusion levels possible to use without compromising physical pellet quality was determined. The suggested optimums were found on the edge of the of the binary tunicate and FM blends, ranging from EX4 to the blend of 220 g/kg DM tunicate, 125 g/kg DM FM and 0 SPC g/kg DM (Tables 2 and 3, Fig. 3E). A tunicate level of 220 g/kg DM correspond to 155 g/kg of tunicate meal in a final coated feed with 32% lipid content. As discussed in Samuelsen et al. (2018), pellet expansion and physical quality can also be improved by change in extrusion parameters as water, steam and mechanical energy. Level and in-between variation of the used ingredients can also impact the results. Due to the chosen moisture content, there is a negative effect on physical feed quality with increased SPC in the blends (Confer 4.2) and consequently no optimum levels for the replacement of SPC by tunicate were found. This design was chosen in order to produce measurable and satisfying pellet quality in all the design points. Recommendation on replacement levels of SPC by tunicate have therefore to be found in other studies where extruder setup and conditions are optimised for SPC extrusion.

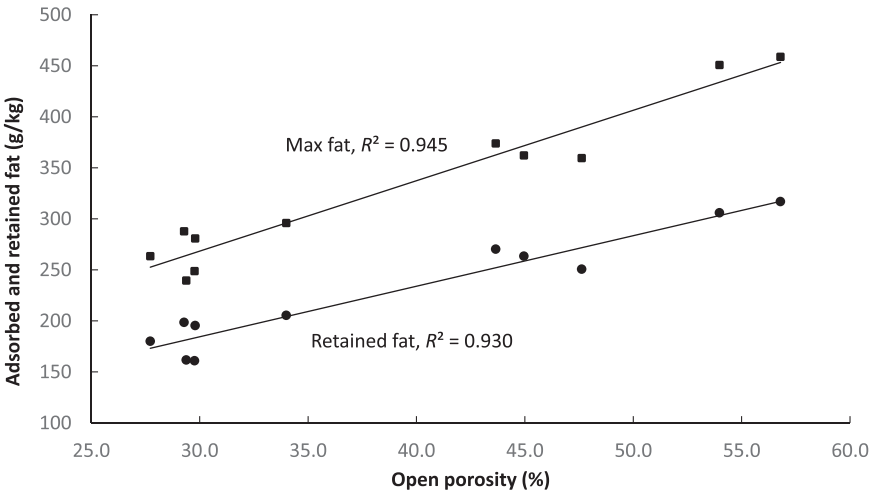


**Fig. 5.** Adsorbed (Max fat) and retained fat as a function of open porosity for the pellet samples.

## 5. Conclusion

The ratio between the protein ingredients tunicate, FM and SPC in the 3-component simplex-centroid mixture design had significant impact on the measured extruder, pellet, and microstructure responses. Increase in tunicate level significantly reduced the feed mass viscosity in the extruder die resulting in increased volumetric expansion and open porosity. Extrudate expansion, fat adsorption capacity and leakage were explained by the extrudate microstructure measured by X-ray microtomography. Within the boundary of the chosen design, 64% of the FM can be replaced by tunicate meal without compromising physical feed quality. Due to its viscosity behaviour, tunicate meal can improve pellet expansion and fat absorption capacity of extruded feed.

## CRediT authorship contribution statement

**T.A. Samuelsen:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Supervision. **G. Haustveit:** Formal analysis, Investigation, Writing – review & editing, Visualization. **K.Kousoulaki:** Conceptualization, Methodology, Writing – review & editing, Project administration, Funding acquisition.

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