# Fucoidan hydrogels significantly alleviate oxidative stress and enhance the endocrine function of encapsulated beta cells

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**Abstract**

Microencapsulating pancreatic islets in immunoprotective hydrogels is a promising cellular therapy for type 1 diabetes. However, a major factor limiting the encapsulated islet efficacy is inflammatory/hypoxia mediated oxidative stress, resulting in impaired insulin secretion and ultimately islet cell death. Fucoidan, a natural polysaccharide, possess strong anti-oxidant properties but its effects on beta cells and encapsulation is unknown. Here, we assessed the anti-oxidant effect of fucoidan on beta cells and its effect on encapsulated beta cell viability and function, using fucoidans extracted from two different seaweeds, namely *Fucus vesiculosus* (FF) and *Ascophyllum nodosum* (FA). FF exhibited significantly higher total antioxidant capacity and free radical scavenging activity, significantly alleviating intracellular oxidative stress in INS1E beta cells, when compared to FA. In presence of high glucose, FF fucoidans significantly increased insulin secretion both in a dose- and time-dependent manner. Viability, ATP levels and high-glucose responsiveness of rat islets encapsulated in fucoidan-containing hydrogel (Fucogel) microcapsules were significantly higher compared to those encapsulated in pure alginate microcapsules. Similar results were obtained with INS1E pseudo-islets and neonatal pig islets. Fucogels can provide a redox-modulatory niche and an immune barrier in the same time, presenting as an outstanding biomaterial for bioengineered immunoprotective beta cell replacement devices.

## 1. Introduction

Type 1 diabetes (T1D) is an autoimmune disorder, hallmarked by the specific destruction of the insulin producing beta cells in the pancreatic islets leading to poor blood glucose control. Type 1 diabetes is typically treated by exogenous insulin administration (either through injections or pumps), accompanied with regular blood glucose checks via fingerpricks multiple times a day. Although most patients can cope relatively well with their diabetes,

exogenous insulin can not fully compensate for the natural blood glucose regulation by beta cells, wich ultimately leads to long term complications, such as vascular damage, neuropathy, loss of sight and kidney failure [1]. The best way to prevent these complications, and restore the natural blood glucose homeostasis is by replacing the patient's lost beta cell mass. Pancreatic islet transplantation, or clinical islet transplantation, is an interesting treatment option for patients with severe diabetes, which involves the infusion of human donor islets into the hepatic portal vein [2]. Although effective, the intervention is not very efficient, ~ 60% of the islets are lost in the first two weeks after transplantation. This is caused by a combination of different factors, lack of revacularisation, high concentrations of lipids and glucose, an immediate blood mediated inflammatory response, allo- and auto-immune rejection and high concentrations of immunosuppressive drugs, causing stress in beta cells [2]. In most cases the recipient needs to be transplanted multiple times before true insulin independence is achieved. Due to the harsh immunosuppresive therapy accompanying this intervention only the severly affected, or patients who have received a kidney transplant, are deemed eligble for clinical islet transplantation at this moment.

A very interesting strategy to omit the use of immunospressive drugs is to encapsulate the donor islets in an immunoprotective envelope to block the recipent's immune cells from directly interacting with the donor cells. The envelope typically consists of a nanoporous membrane or a hydrogel, such as alginate, in which the pancreatic islets are embedded prior to transplantation. Alginate microcapsules allow for the diffusion of nutrients, oxygen and metabolites to and from the encapsulated islets but, also provide an immunoprotective barrier preventing the interaction of immune cells and diffusion of large molecular weight antibodies [3]. Considering their large volume, microencapsulated islets have been mostly transplanted into the peritoneal cavity and have been shown to reverse diabetes in various preclinical small and large allo- and xeno- transplantation animal models with variable success rates [4]. Phase 1 clinical trials demonstrated that the use of microencapsulated human islets was safe but, could

only provide a minor clinical benefit due to poor graft survival [5,6]. The most important factors contributing to encapsulated islet graft failure are, lack of oxygen and a foreign body response to the implant causing fibrosis, leading to hypoxia mediated cell damage and to long term graft failure [3]. Native islets residing within the pancreas are highly vascularized microtissues, surrounded by a acinar cells and a specialized extracellular matrix, and receive ample oxygen ($pO_2$ of 40 mmHg) and nutrient supply [7]. During the isolation process this dense vascular network is destroyed, and the islets are removed from their pancreatic niche leading to significant post-isolation cell stress. This is further aggrevated by the encapsulation process, which prevents revascularization, and moreover, we have recently shown that different biomaterials can induce significant amounts of oxidative stress in both beta and alpha cells [8]. The absence of revascularization and limited mass transport caused by the immunoprotective barrier subject the encapsulated islets to irreversible chronic hypoxia induced oxidative stress. Although microencapsulation can protect against immune cells and large antibodies, the islets are still vulnerable to smaller molecules such as chemokines, cytokines and reactive oxygen species (ROS), which can easily permeate through the microcapsule pores [9]. Some of these small molecules are also produced by islets themselves in response to various stress stimuli further aggrevating the unfavorable conditions [10]. It has been shown that ROS such as $H_2O_2$ and superoxide radicals, can not only negatively impact insulin secretion, but also activate beta cell specific autoreactive T cell subsets, which play an important role in beta cell destruction [11–14]. Although the mechanisms leading to encapsulated graft failure is not fully understood, it is clear that hypoxia and inflammation induced oxidative stress can have a significant impact on beta cell survival and function. Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species and antioxidant defences in the cell leading to tissue damage [15]. It has been shown that beta cells are highly susceptible to oxidative stress and vulnerable to ROS mediated damage due to their low intrinsic intracellular levels of antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (Gpx-1)

[16]. Strategies to mitigate oxidative stress should improve encapsulated islet function and survival and potentially lead to better *in vivo* outcomes.

Several strategies have been investigated to protect encapsulated islets from oxidative stress induced cell death. It has been shown that entrapping a natural antioxidant curcumin within a polymer matrix can reduce oxidative stress and support insulin secretion in encapsulated rat islets [17]. Studies have demonstrated the benefit of incorporating tannic acid in microcapsules to scavenge ROS and suppress the production of pro-inflammatory cytokines leading to improved encapsulated islet survival and function [18,19]. The technique of incorporating antioxidants into microcapsules allows for local delivery of redox-modulating molecules directly to the islets at the site of transplantation without the need for systemic administration. In the last decade a number of different brown seaweed species have been described to posses high antioxidant capacity [20]. Their potent antioxidant capacity is attributed to a wide variety of polymers and small molecules in these seaweeds of which a particular group of sulfated polysaccharides named fucoidans are the most important. The molecular weight of fucoidan influences its hydroxyl radical scavenging activity and reduction potential [21]. Apart from their anti-oxidant activity, fucoidans also possess anti-inflammatory properties, and are known to suppress mechanistic events of inflammation such as activation of NF-kβ, generation of reactive oxygen species (ROS), upregulation of proinflammatory cytokines and associated cell adhesion molecules [22].

Here we report about the effect of fucoidan on islets and beta cells when used in a microencapsulation strategy. We compared fucoidans obtained from two different algae species *Fucus vesiculosus* (FF) and *Ascophylum nodosum* (FA) (with varying molecular weights) and determined their potency to alleviate oxidative stress *in vitro*. We show that fucoidan obtained from FF has high antioxidant capacity, but is also able to significantly decrease oxidative stress in islets and beta cells when cultured in oxidative stress conditions. In addition, incorporation of FF fucoidan in alginate microcapsules has no detrimental effect on mass transport or the

mesh size of ultrapure alginate hydrogels, and leads to a significant increase in cell survival and insulin secretion.

## 2. Results & Discussion

### 2.1. Characterization and antioxidant properties of fucoidans

Seaweeds growing in shallow waters are continuously exposed to ultraviolet light and air, which leads to the formation of free radicals and other reactive oxygen species (ROS). Despite chronic exposure to ROS, healthy seaweeds are resilient to oxidative damage demonstrating the existence of potent antioxidant defense mechanisms within their cells that is attributed to sulphated polysaccharides namely fucoidans [21]. Fucoidans constitute the cell walls of these brown seaweeds and have a very complex chemical composition of which sulphated-l-fucose is the major component [23]. The biological activity of fucoidan is largely determined by these sulphate groups and is highly variable between the different genera of brown seaweeds and the extraction procedure [23]. In this study, we characterized the fucoidans extracted from two brown seaweed genera *Fucus vesiculosus* (FF) and *Ascophyllum nodosum* (FA) and determined their antioxidant potential.

NMR spectra of both fucoidans (FA & FF) show similar general bands **(Figure S1)**. NMR spectral bands typical of anomeric protons H-1 from α-L-fucose residues and uronic acid residues can be observed between 5.6 and 5.20 ppm. H-2, H-3 and H-4 of sulfated fucose can be found between 4.66 and 4.10 ppm, and bands between 4.20 to 3 ppm typical for H-2, H-3, H-4 and H-5 of unsulfated fucose and uronic acid [24]. The two peaks, appearing around 1.2 and 1.35 ppm were assigned to the methyl group belonging to fucose. We also observed an individual peak at 2.2 ppm, which can be ascribed to acetyl groups.

Monosaccharide analysis of the purified fucoidans (FF & FA) **(Figure 1A)** showed that the predominant sugar moiety present in both batches was fucose (18% - 38% w/w) followed by xylose (4-5% w/w), galactose, mannose rhamnose (1-2% w/w) and traces of glucose and

arabinose (< 1% w/w). Fucose content was found to be significantly higher in FF compared to FA fucoidan, while other carbohydrate species were similar. Similar to fucose sugars, sulfate quantification analysis demonstrated that FF fucoidan has a significantly higher sulfate content (30 ± 4.4%) compared to FA fucoidan (20 ± 3.7%) (Figure 1B). Chemical characterization of both FF and FA fucoidans demonstrated the presence of varied sugar moieties (rhamnose, fucose, ribose, arabinose, xylose, mannose, galactose and glucose) and as well uronic acid and sulfate groups as reported in the literature [25–27]. However, in this study, we found that fucose sugars and sulfate content is significantly higher in FF compared to FA fucoidan. The difference in the fucose sugars and sulfate composition determine the antioxidant capacity of fucoidan and it varies according to the algal species, the season when they are collected and the method used for the extraction procedure [28,29].

The antioxidant potential of purified FF and FA fucoidans was determined using the total antioxidant capacity assay kit and expressed as TEAC with Trolox serving as an antioxidant standard. As seen from Figure 1C, both FA and FF fucoidans demonstrated a dose-response increase in their antioxidant capacity (measured as TEAC) with FF fucoidan always possessing significantly higher amount of antioxidants than FA fucoidan at all tested concentrations. Subsequently, the concentration of antioxidant (measured as Trolox equivalents) in FF fucoidan (17.97±1.06 nmoles/µl Trolox equivalents) was ~ 33- fold higher compared to FA fucoidan (0.55±0.06 nmoles/µl Trolox equivalents) (Figure 1D). The high antioxidant capacity of FF fucoidan compared to FA may be attributed to its high fucose and sulphate content [28,29].

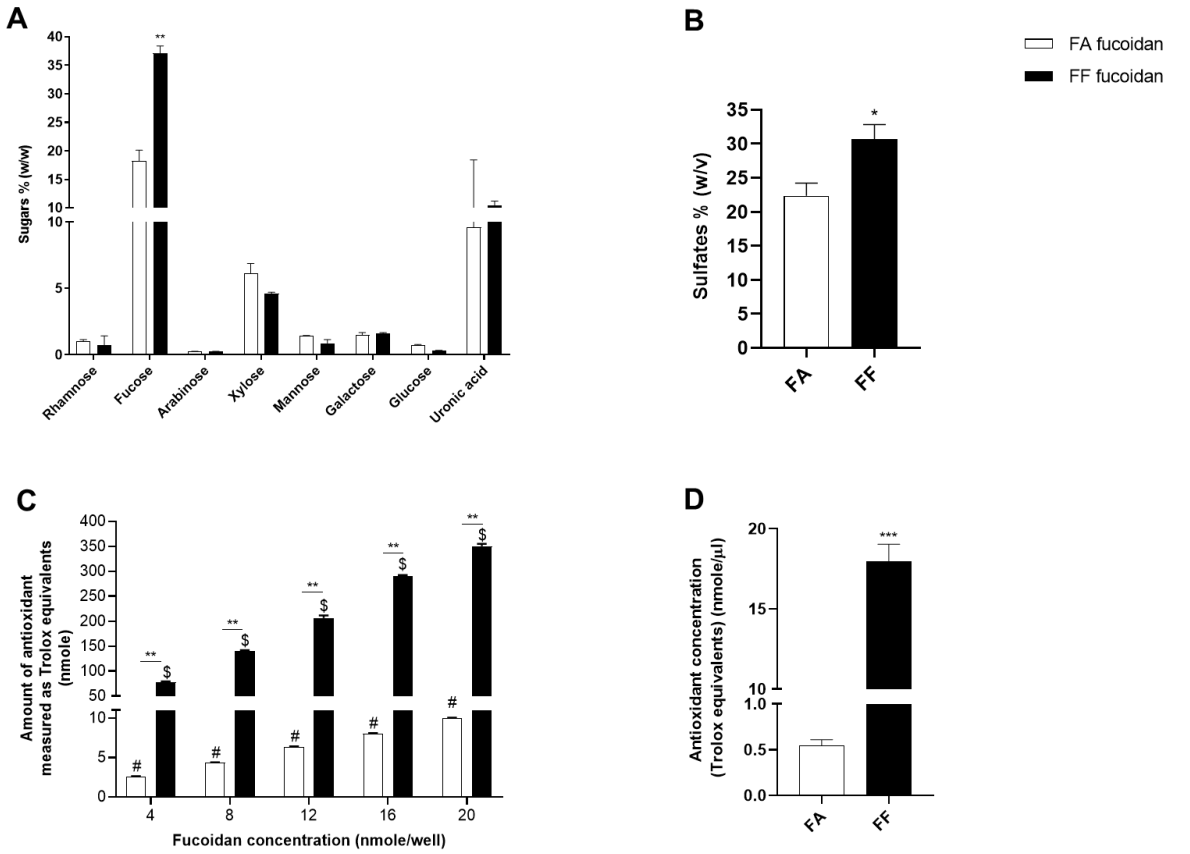


**Figure 1.** Chemical characterization and antioxidant properties of purified fucoidans. Sugar composition of purified FF & FA fucoidans obtained from different species; Values = mean ± SEM; $^{**}p < 0.01$ when compared FA and FF fucoidans (Student's t-test) (A). Sulfation degree (%) of purified FF & FA fucoidans (B); Values = mean ± SEM; $^{*}p < 0.05$ when compared between FF and FA fucoidans (Student's t-test). Measurement of the total antioxidant capacity of both FF and FA fucoidans measured as Trolox equivalents (C). Trolox, a vitamin E analogue was used as a standard. Values = mean ± SEM (n=6); $^{\#,\$}p < 0.0001$ for the amount of antioxidant seen with different concentrations for both FF and FA fucoidans (ANOVA with posthoc Dunnett's T3 multiple comparisons test and $^{**}p < 0.01$ for the amount of antioxidant when compared between FF and FA fucoidans at different concentration (Student's t-test). Concentration of antioxidant measured as Trolox equivalents between FF and FA fucoidans (D). Values = mean ± SEM (n=6); $^{***}p < 0.0001$ when compared between FF and FA fucoidans (Student's t-test).

**2.2 Cytotoxicity and free radical scavenging activity of fucoidans and its potential to alleviate oxidative stress**

INS1E cells were used to evaluate the cytotoxicity of varied fucoidans in this study as it is a model insulin-producing cell and further studies were carried out using this cell line. The toxicity of both FF and FA fucoidans was determined using a direct contact cytotoxicity assay. To evaluate the cytotoxicity and optimize the fucoidan concentration, INS1E cells were seeded into 96-well plates, treated with varied concentrations of both FF and FA fucoidans for 24 h and an MTT assay was performed. Both FF and FA fucoidans at concentrations ranging from 2.5 mg $ml^{-1}$ to 0.15 mg $ml^{-1}$ were found to be nontoxic with a cell viability of ≥ 90% similar to the control cells indicating acceptable biocompatibility **(Figure 2A)**. However, at concentrations of 5 and 10 mg $ml^{-1}$, both FF and FA fucoidans demonstrated a slight decrease in cell viability. Regardless, both FF and FA fucoidans was found to be non-toxic and did not elicit a cytotoxic response with cell viabilities well above the cut-off value of 70% for all dilutions tested (Figure 2A).

To determine the free radical scavenging activity of the different fucoidans, a DPPH free radical assay was carried out, and the ability of fucoidans to scavenge DPPH free radical was determined. Generally, a high percentage of DPPH free radical inhibition at low $IC_{50}$ values indicates a high antioxidant activity. As seen from Figure 2B, FF fucoidan above 7.8 µg $ml^{-1}$ exhibits a dose response increase in their free radical scavenging activity with a maximum inhibition of 78.6 ± 0.66% seen at 1000 µg $ml^{-1}$ with an $IC_{50}$ of 362.2 µg $ml^{-1}$. The standard ascorbic acid at the same concentration (1000 µg $ml^{-1}$) exhibited a maximum inhibition of 94.7 ± 0.21% with a low $IC_{50}$ of 6.2 µg $ml^{-1}$ (Figure 2B). In contrast, FA fucoidan showed very poor free radical scavenging activity compared to FA fucoidan with a maximum inhibition of only 8.7 ± 0.8% seen at 1000 µg $ml^{-1}$. This clearly shows that FF fucoidan exhibits significantly higher free radical scavenging capacity compared to FA fucoidan. One factor that largely

determines the reducing ability of fucoidan is its molecular weight. It has been reported that low molecular weight fucoidan possesses significantly higher antioxidant and radical scavenging properties compared to high molecular weight fucoidan as it can easily cross the lipid bilayer and exert a biological effect [24,30]. In contrast, we found that the relatively high molecular weight FF fucoidan (91 kDa) exhibits superior free radical DPPH scavenging activity, compared to the lower molecular weight FA fucoidan (5-15 kDa), suggesting its strong free radical scavenging capacity. This discrepancy can be explained by other factors such as degree of substitution groups and their position, and glycosidation branching, which are known to influence the reducing properties of fucoidan [31,32].

Subsequently, to determine the biological potential of fucoidans in alleviating oxidative stress, an intracellular oxidative stress assay was carried out in INS1E beta cells using a 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA) that de-esterifies intracellularly and turns into highly fluorescent 2′,7′-dichlorofluorescein upon oxidation by a wide range of reactive oxygen species (ROS) including $H_2O_2$ [33]. The amount of fluorescence signal is directly proportional to the intracellular oxidative stress levels and can be used to determine the effect on ROS in cells during exposure of the different fucoidans. Pre-treatment with both FF and FA fucoidans reduced the basal oxidative stress levels in INS1E cells compared to control though not significant (Figure 2C). However, it can be seen that in the presence of $H_2O_2$, the intracellular oxidative stress levels in INS1E cells was significantly alleviated in cells pre-treated with FF fucoidan compared to $H_2O_2$ treated cells and as well lower than the positive control N-acetyl cysteine (NAC) treated cells suggesting strong ROS mitigating activity. On the contrary, FA fucoidan did not exhibit any ROS mitigating activity in alleviating oxidative stress, and intracellular oxidative stress levels were similar to control cells treated with $H_2O_2$ alone (Figure 2C). Apart from its strong free radical scavenging activity, we observed that FF fucoidan significantly reduces intracellular oxidative stress levels in beta cells exposed to $H_2O_2$ compared to FA fucoidan. This beneficial effect may be attributed to the redox modulating property of FF

fucoidan by the induction of the antioxidant enzymes leading to protecting of the beta cells from free radical mediated toxicity [34]. Thus, the significantly higher antioxidant concentration and free radical scavenging activity of FF compared to FA fucoidan clearly demonstrate its superior antioxidant capacity to alleviate oxidative stress and hence used in subsequent studies.

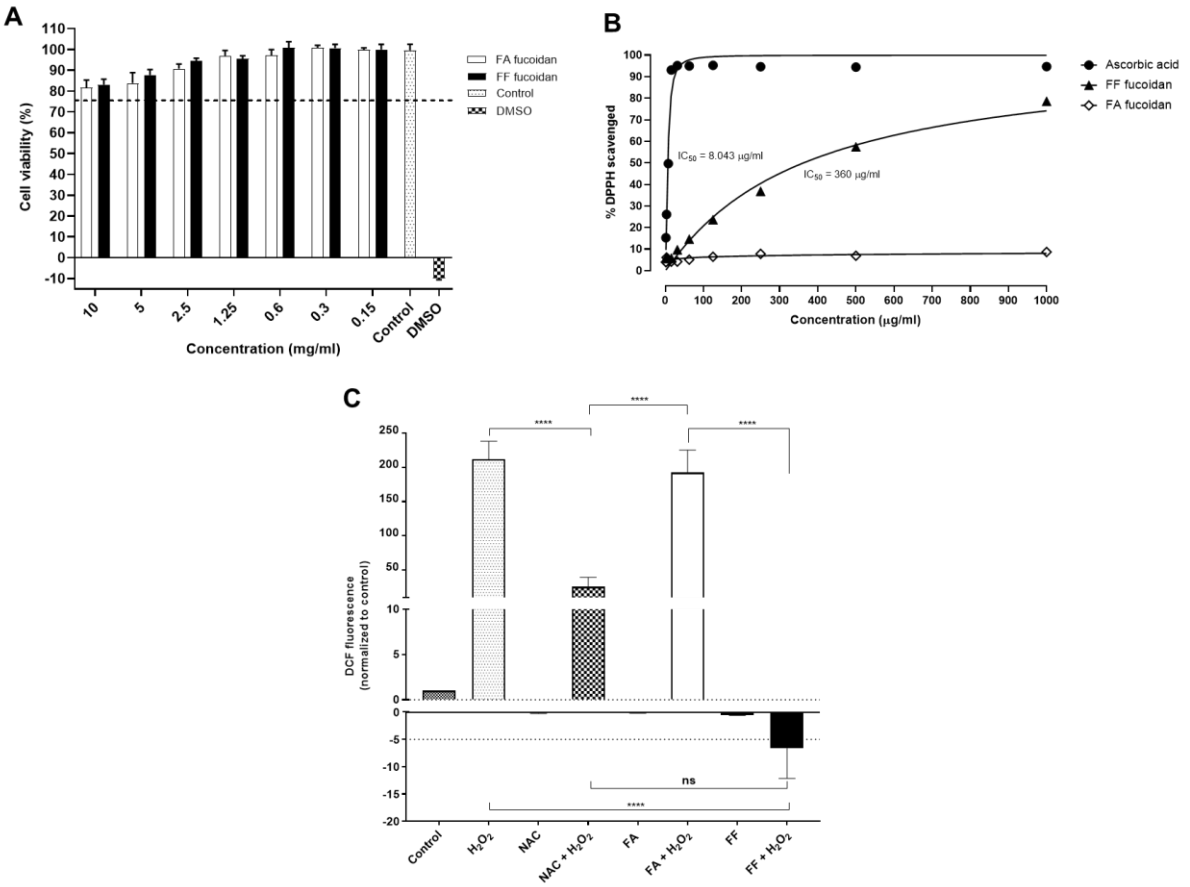


**Figure 2.** Cytotoxicity and free radical scavenging properties of fucoidan and its ability to alleviate oxidative stress: The cytotoxicity of fucoidans was determined in a MTT assay using INS1E cells (A). Values = mean ± SEM (n=3); $p > 0.05$ (ANOVA with posthoc Dunn's multiple comparisons test). DPPH free radical scavenging activity of FF and FA fucoidans (B). Values = mean ± SEM (n=3). Effect of FF and FA fucoidans to alleviate intracellular oxidative stress in INS1E cells using the DCFH-DA probe (C). In the presence of $H_2O_2$, FF fucoidan significantly alleviated oxidative stress compared to FA fucoidan. Values = mean ± SEM (n=3);

****p<0.0001 when compared FF and FA with control NAC in the presence of $H_2O_2$ (ANOVA with posthoc Dunnett's multiple comparisons test).

### 2.3 Effect of fucoidan on beta cell viability and function

Apart from its antioxidant properties, fucoidan has been shown to possess anti-diabetic properties by inducing insulin secretion, and reducing blood glucose levels, stimulating glucose uptake and improving insulin sensitivity [35–37]. To explore the direct effect of FF fucoidan on insulin secretion, INS1E cells were treated with varying doses of fucoidan and for different time intervals. **Figure 3A**, shows that incubation of INS1E cells with different FF fucoidan concentrations (0.01, 0.1, 0.5, 1, 5 and 10 mg $ml^{-1}$) for 3 hr induced insulin secretion in a dose-dependent manner with a significant increase seen with 5 and 10 mg $ml^{-1}$ compared to high glucose. However, there was no significant difference in the insulin induction between 5 and 10 mg $ml^{-1}$ FF fucoidans. Therefore, for time-response studies, we chose 5 mg $ml^{-1}$ of FF fucoidan and treated the INS1E cells with this concentration for 6, 12 and 24 hr respectively. At all time points tested, FF fucoidan treatment significantly increased insulin secretion in a time-dependent manner in the presence of high glucose (Figure 3B). This data demonstrates the insulinogenic properties of FF fucoidan and that the FF induced insulin secretion is both dose- and time-dependent.

Further to determine the effect of fucoidan on primary beta cells, rat islets were treated with FF fucoidan (1 mg $ml^{-1}$) for 24 hr and their viability and function assessed. Pre-treatment with FF fucoidan significantly improved rat islet viability and function. Live/dead analysis revealed that the viability of islets treated with FF fucoidan was significantly higher compared to non-treated controls (Control vs FF treated: 84.5 ± 1.6% vs 93.1 ± 1.9%; $p < 0.05$) (Figure 3C). When comparing function in static glucose stimulated insulin secretion (GSIS), rat islets treated with FF fucoidan showed a significantly better insulin secretory behaviour, with higher insulin release in response to glucose stimulation compared to non-treated controls (Figure 3D).

This improved function resulted in a significantly higher stimulation index (20.1 ± 2.8) which is ~ 1.8 fold higher in FF treated islets compared to non-treated controls (11.1 ± 0.7) (Figure 3D).

The above data suggest that islets pre-treated with FF fucoidan responded better to changes in glucose with significantly higher insulin secretion and improved stimulation indices. We also showed that in high glucose conditions, FF fucoidan support the increase of insulin secretion in a dose- and time- dependent manner in comparison to controls. FF fucoidan assisted insulin secretion may be attributed to an increase in cAMP concentrations which has shown to be significantly elevated in fucoidan treated insulin producing RIN-5F cells [37]. Elevation in cAMP levels has been shown to stimulate insulin exocytosis both in a $Ca^{2+}$ dependent and independent mechanisms and supports membrane depolarization by directly inhibiting $K_{ATP}$ channels [38–40].

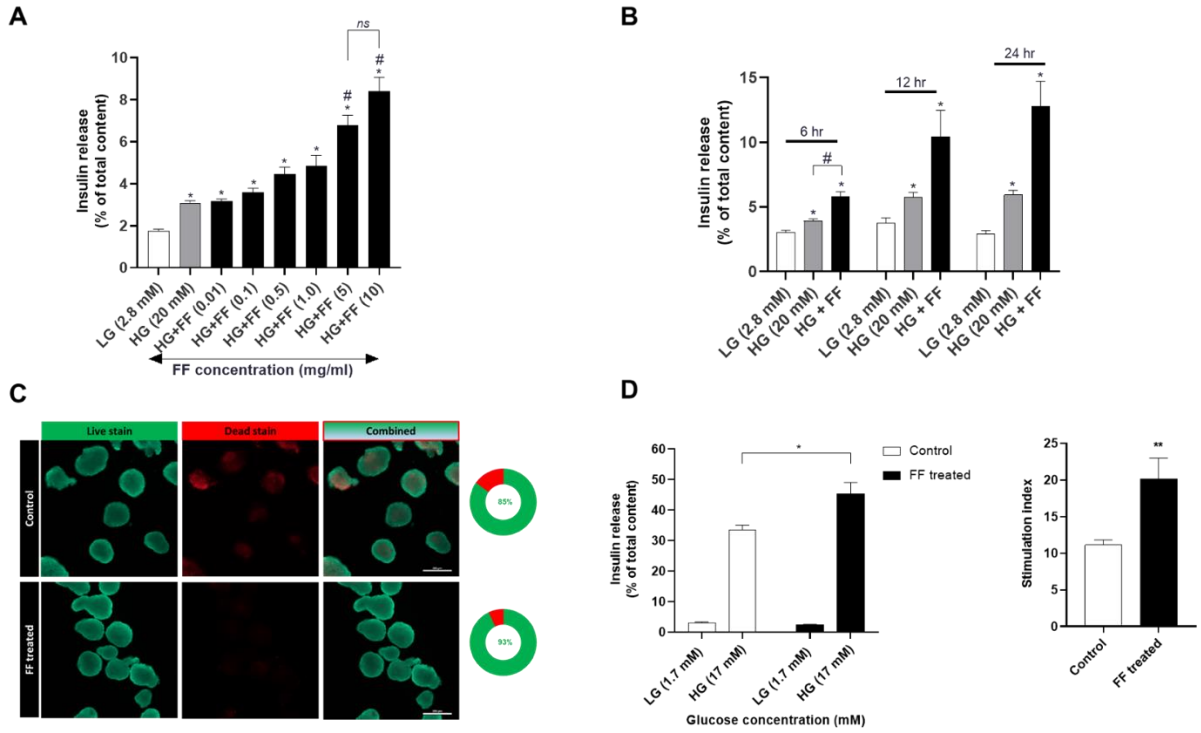


**Figure 3.** Effect of fucoidan on beta cell viability and function: Measurement of insulin secretion in INS1E cells exposed to varied concentrations of FF fucoidan in the presence of

high glucose (HG) for 3 hr (A). Incubation of INS1E cells with different FF fucoidan concentrations (0.01, 0.1, 0.5, 1, 5 and 10 mg $ml^{-1}$) induced insulin secretion in a dose-dependent manner compared to low glucose (LG) ($^{*}p<0.0001$) and with a significant increase seen with 5 and 10 mg $ml^{-1}$ compared to high glucose ($^{\#}p<0.001$) (ANOVA with posthoc Dunnett's T3 multiple comparisons test). Measurement of insulin secretion in INS1E cells exposed to 5 mg $ml^{-1}$ of FF fucoidan in the presence of high glucose (HG) for 6, 12 and 24 hr (B). Values = mean ± SEM (n=3). In the presence of high glucose (HG), at all-time points tested, FF fucoidan treatment significantly increased insulin secretion in a time-dependent manner compared to low glucose (LG) ($^{*}p<0.05$) and with a significant increase seen with high glucose (HG) at 6 hr time point (#p<0.05) (ANOVA with posthoc Dunnett's T3 multiple comparisons test). Viability of primary rat islets pretreated with FF fucoidan for 24 hr (C). Pretreatment with FF fucoidan significantly improved islet viability (93.1 ± 1.9%) compared to the non-treated controls (84.5 ± 1.6%) ($p<0.05$) (Student's t-test). Values = mean ± SEM (n=25 islets). Assessment of primary rat islet function in glucose stimulated insulin secretion (GSIS) (D). Pretreatment with FF fucoidan significantly improved islet function ($^{*}p<0.05$) which resulted in a significantly higher stimulation index ($^{**}p<0.01$) compared to non-treated controls.

**2.4 Characterization of fucoidan hydrogels and fucoidan microcapsules**

Based on the above positive findings we then explored the possibility of incorporating FF fucoidan in ultra-pure alginate microcapsules as a carrier for beta cells and islets. We chose ultrapure alginate since it is used in the field of type 1 diabetes for a long time and has an extensive track record in microencapsulation of islets [3]. Fucoidan is a water-soluble molecule with low viscosity and cannot be used by itself for cell microencapsulation purposes. However, by mixing fucoidan with alginate we were able to obtain a suitable carrier for cell microencapsulation using an air driven microdroplet generator. In brief, FF fucoidan (0.5% w/v) was mixed with ultrapure sodium alginate (2% w/v) to obtain a 2.5% w/v alginate/fucoidan

polymer solution that is then cross-linked with barium chloride (20 mM) to form alginate/fucoidan hydrogels. The alginate/fucoidan hydrogel will hereafter be referred to as "Fucogel" and alginate/fucoidan microcapsules as "Fucocaps". The antioxidant potential of fucogel, and alginate alone, was determined using a total antioxidant capacity assay kit. Both alginate and fucogel demonstrated a dose-response increase in their antioxidant capacity (measured as TEAC), with fucogel always exhibiting significantly higher amount of antioxidant capacity than alginate alone **(Figure 4A)**. We found that the antioxidant concentration (measured as Trolox equivalents) in fucogel was ~35- fold higher compared to alginate polymer (4.2 ± 0.2 nmoles $\mu l^{-1}$ Trolox equivalents vs 0.12 ±0 .04 nmoles $\mu l^{-1}$ Trolox equivalents) (Figure 4B). The high antioxidant activity of fucogel is due to the FF fucoidan molecules that can be attributed to the high fucose and sulphate content (Figures 1 A&B).

Next, we determined the viscoelastic properties of fucogel using a rheometer. Mechanical testing revealed that both alginate and fucogel have similar viscoelastic properties and show similar strain-dependent behaviour for G' (Figure 4C). Both hydrogels showed a linear viscoelastic region until 0.5% strain. The G' at 0.1% strain was used to calculate their respective hydrogel mesh sizes. There were no significant differences in hydrogel mesh sizes between alginate, 8.15 ± 0.92 nm, and fucogel, 8.05 ± 0.64 nm (Figure 4D). Strain sweep experiments for both hydrogels displayed a stable G' within the linear viscoelastic region (LVR) (until 0.5% strain) (Figure 4C), which is in agreement with literature [41]. Decrease in G' outside of the linear viscoelastic region (LVR), indicates structural breakdown and yielding of the hydrogel microstructure. The G' value within the LVR was subsequently used to calculate the hydrogel mesh size ($D_{mesh}$) which represents the diameter of a hard sphere that fills the void in between hydrogel network strands, and is an important indication for hydrogel diffusion properties. The $D_{mesh}$ of alginate hydrogels has been described to range between 5-90 nm, which seems to be in agreement with our data with a mesh size of 8.15 ± 0.92 nm (Figure 4D) [42–45]. The $D_{mesh}$ of alginate hydrogels depends on its molecular weight, guluronic acid/mannuronic

acid (G/M ratio) and purity and as well the crosslinker concentration. The dimensions of insulin ranges between 2.7 and 5.5 nm and should be capable to diffuse through these alginate hydrogels given that the mesh size is around 8 nm [46]. The ultrapure alginate used in this study has a molecular weight > 200 kDa, while FF fucoidan has a molecular weight of ~ 91 kDa. Hence, the relatively small fucoidan moieties did not hinder the formation of an alginate network, depicted by the similar G' values with $D_{mesh}$ of 8.05 ± 0.64 nm compared to alginate alone hydrogel. This data suggests that fucoidan moieties can be easily blended with alginate hydrogels without compromising the viscoelastic and diffusion properties to form stable fucogels suitable for encapsulation studies.

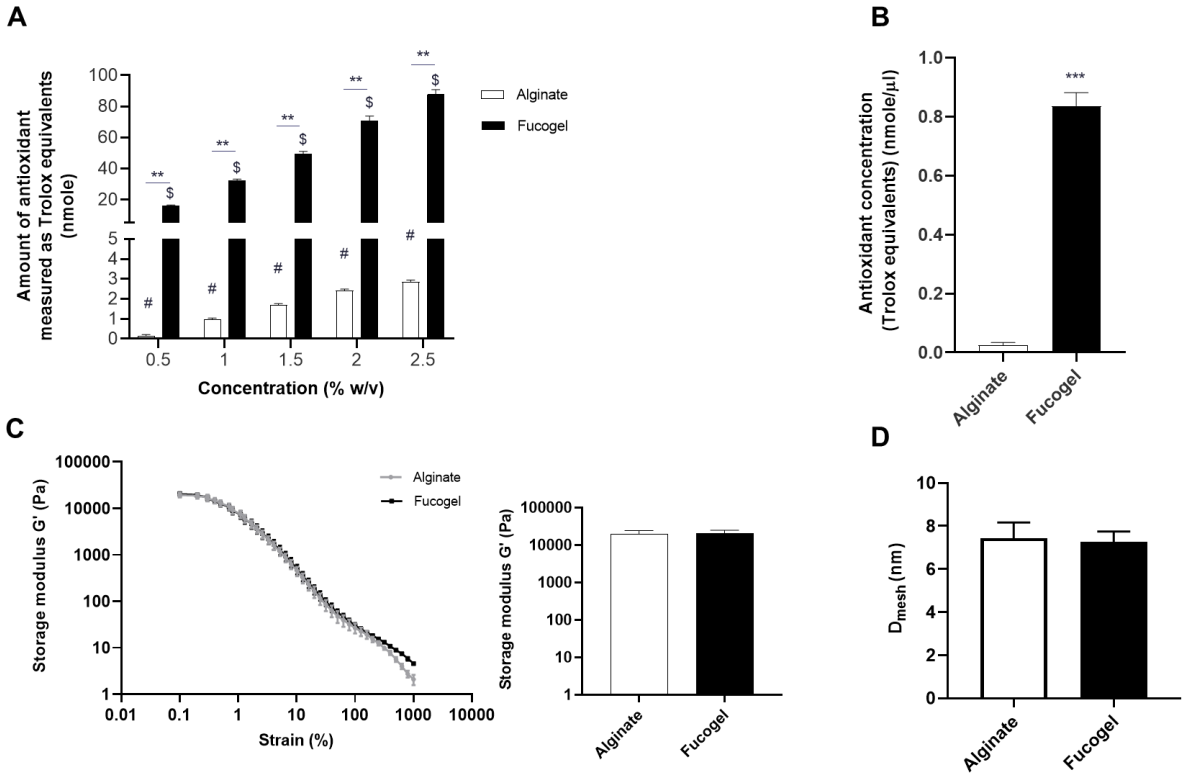


**Figure 4.** Characterization of fucogels: Measurement of the total antioxidant capacity of fucogel measured as Trolox equivalents (A). Trolox, a vitamin E analogue was used as a standard. Values = mean ± SEM (n=6); $^{\#,\$}$ $p<0.0001$ for the amount of antioxidant capacity seen with different concentrations for both fucogel and alginate (ANOVA with posthoc Dunnett's

T3 multiple comparisons test) and $^{**}p<0.01$ for the amount of antioxidant when compared between fucogel and alginate at different concentrations (Student's t-test). Concentration of antioxidant measured as Trolox equivalents between fucogel and alginate (B). Values = mean ± SEM (n=6); $^{***}p<0.0001$ when compared between fucogel and alginate (Student's t-test). Rheological properties of fucogel and alginate: Strain sweep alginate (grey) and fucogel (black) (C) and measurement of hydrogel mesh size based on the storage moduli at 0.1% strain (D) and calculated using formula 1: $D_{mesh} = ((6\ RT)/(\pi N\_av\ G'))^{\wedge}(1/3)$. Values = mean ± SEM (n=5).

Alginate microcapsules and fucocaps were produced using an air-driven droplet generator and their size was determined by phase contrast light microscopy. Light micrographs of alginate and fucocaps show that for both hydrogels the microcapsules are homogenous in size and equal in appearance. Both alginate and fucogel microcapsules have similar dimensions and are composed of uniform spheres of approximately 500 µm (500 ± 24.2 µm) diameter **(Figure 5A)**. The droplet sizes were reproducibly made and the polydispersity in the microcapsule diameter is close to the range typically seen for microcapsules prepared by the air-stripping technique. In order to visualize the presence of fucoidan in fucocaps, microcapsules were stained with TBO and imaged at subsequent regular time intervals for 40 days. After removing excess TBO staining, a clear blue coloration of fucocaps could be seen which persisted during 40 days. In Figure 4A, clear toluidine blue staining can be seen after 40 days indicative of the long term presence of fucoidan in fucocaps. These data suggest that the fucocaps remained stable, while toluidine blue staining confirmed the presence of fucoidan within these microcapsules for at least 40 days suggesting the entrapment of small fucoidan moieties within the crosslinked alginate network. However, the release kinetics of fucoidan from fucocaps needs to be evaluated in future studies.

Native islets residing within the pancreas are highly vascularized microtissues that are destroyed during the islet isolation process leading to post-isolation cell stress [15]. This is

further aggravated by the encapsulation process which prevents revascularization, and moreover, we have recently shown that different biomaterials can induce significant amounts of oxidative stress in both beta and alpha cells [16]. The absence of revascularization and limited mass transport caused by the immunoprotective barrier subject the encapsulated islets to chronic oxidative stress. Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and antioxidant defenses, and islets are particularly prone to ROS due to their relatively low antioxidant enzymes [2]. Therefore, in this study, we explored the potential of FF fucoidan to alleviate oxidative stress post-encapsulation using a genetically encoded redox probe mt-roGFP2-Orp1 transduced INS1E beta cells. The probe is a fusion protein consisting of redox-active green fluorescent protein 2 (roGFP2) genetically fused to the redox enzyme Orp1 that is oxidized by $H_2O_2$ [17]. A major advantage of roGFPs over other fluorescent probes is their ratiometric fluorescence excitation behavior. When measuring fluorescence emission at 510 nm, roGFP2 exhibits excitation maxima at both 400 and 490 nm; oxidation increases the intensity of the 400 nm peak while decreasing the intensity of the 490 nm peak and vice versa for reduction (Figures 5 B&C). Using the ratiometric fluorescence, we found that INS1E beta cells in fucocaps experienced significantly less oxidative stress compared to alginate microcapsules (Figure 5D). Further, exposure of encapsulated INS1E beta cells to $H_2O_2$ (1 mM) for 1 hr, suggested the oxidative stress is significantly reduced in Fucocaps compared to those in alginate microcapsules (Figure 4D). These data again confirm the free radical scavenging capacity of fucoidan and its ability to alleviate oxidative stress post-encapsulation and in the presence of an external stress stimulus.

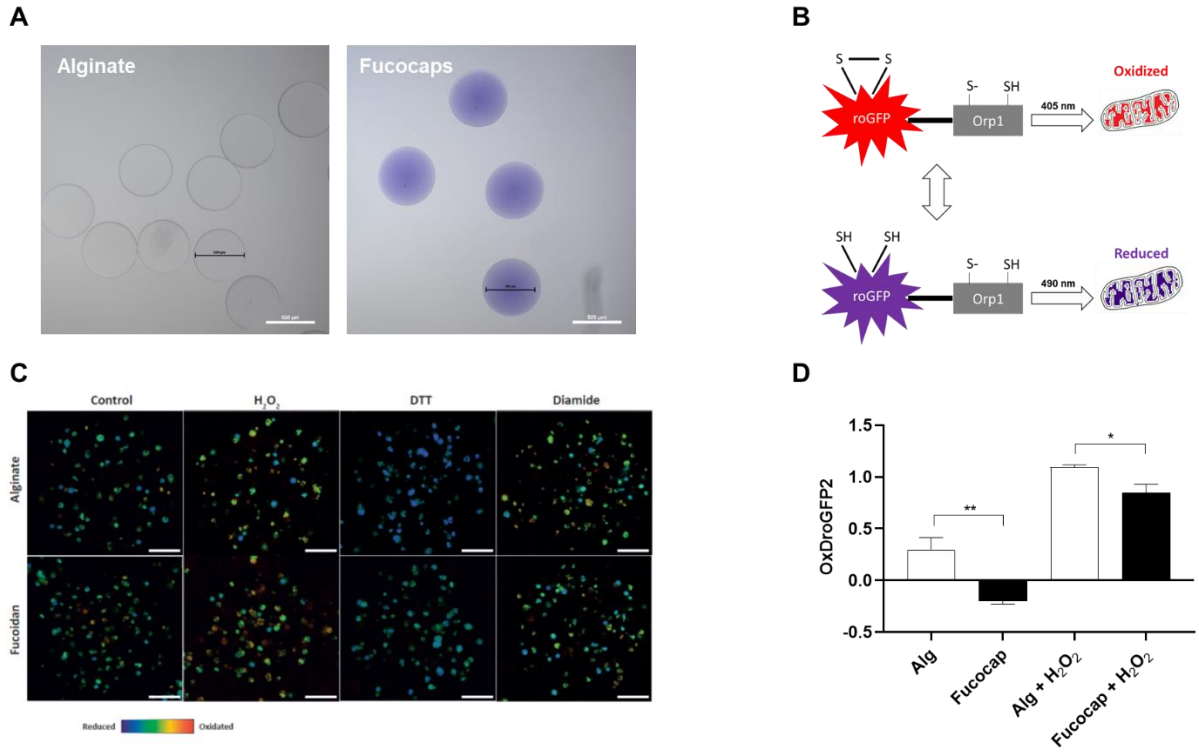


**Figure 5.** Characterization of fucocaps: Optical microscopy images of alginate and fucocaps generated by an air-droplet generator with size of ~500 µm and toluidine blue staining to demonstrate the presence of fucoidan within the fucocaps (A). Schematic illustration of the mitochondrial ROS sensor mt-roGFP2-Orp1 (B). To explore the potential of fucoidan to alleviate oxidative stress post-encapsulation a genetically encoded redox probe mt-roGFP2-Orp1 transduced INS1E beta cells were used. The fluorescence maximum of this sensor shifts from 490 to 400 nm with increasing mitochondrial $H_2O_2$ levels, as shown by representative fluorescence images (C). Ratiometric fluorescence demonstrated that mt-roGFP2-Orp1 transduced INS1E beta cells encapsulated in Fucocaps experienced significantly less oxidative stress compared to alginate microcapsules ($^{**}p<0.01$). Further, exposure of encapsulated mt-roGFP2-Orp1 transduced INS1E beta cells to $H_2O_2$ suggested the oxidative stress is significantly reduced in Fucocaps compared to those in alginate microcapsules (D) ($^{*}p<0.05$) (ANOVA with posthoc Dunnett's T3 multiple comparisons test). Values = mean ± SEM (n=3).

### 2.5 Encapsulation of insulin producing cells in fucocaps

To determine the effect of fucoidan encapsulation on beta cell survival and function both primary rat islets and pseudoislets (generated from rat insulin producing INS1E cell line) were encapsulated in fucocaps, cultured and evaluated at different time points for cell viability, ATP content, and beta cell function by glucose stimulated insulin secretion. Live/dead analysis revealed that the viability of primary rat islets encapsulated in fucocaps was always higher compared to those encapsulated in alginate microcapsules. Viability of islets in fucocaps was significantly higher both on days 1 (95.2 ± 1.4%) and 5 (91.3 ± 2.1%) respectively compared to those in alginate microcapsules (Day 1 vs Day 5: 86.2 ± 3.6 vs 83.5 ± 2.3%) **(Figure 6A)**. This is further confirmed by ATP measurements, whereby the ATP levels of rat islets encapsulated in fucocaps was significantly higher on day 1 and 5, ~ 1.2- fold, compared to islets in alginate microcapsules (Figure 6B). Regarding islet function, rat islets encapsulated in fucocaps showed a better insulin secretory behaviour, including a significantly higher stimulation index at days 1 (59.6 ± 2.4) and 5 (53.3 ± 9.5) compared to alginate controls (Day 1 vs Day 5: 32.6 ± 2.8 vs 28.4 ± 2.1) (Figure 5C). Similar results were found for pseudoislets, whereby INS1E pseudoislets encapsulated in fucocaps demonstrated a significantly higher viability both on days 1 (90.2 ± 4.5%) and 5 (95.5 ± 0.01%) respectively compared to alginate microcapsules (Day 1 vs Day 5: 80.1 ± 0.01% vs 90.2 ± 3.2%) **(Figure S2A)**. The ATP levels of pseudoislets encapsulated in fucocaps was significantly higher on day 1 and 5, 1.5- and 1.4-fold, compared to those embedded in alginate microcapsules (Figure S2B). Further, pseudoislets encapsulated in fucocaps responded better to high glucose stimuli, and with a significantly higher stimulation index on day 5 (2.4 ± 0.2) compared to those encapsulated in alginate microcapsules (1.6 ± 0.1) (Figures S2 C&D).

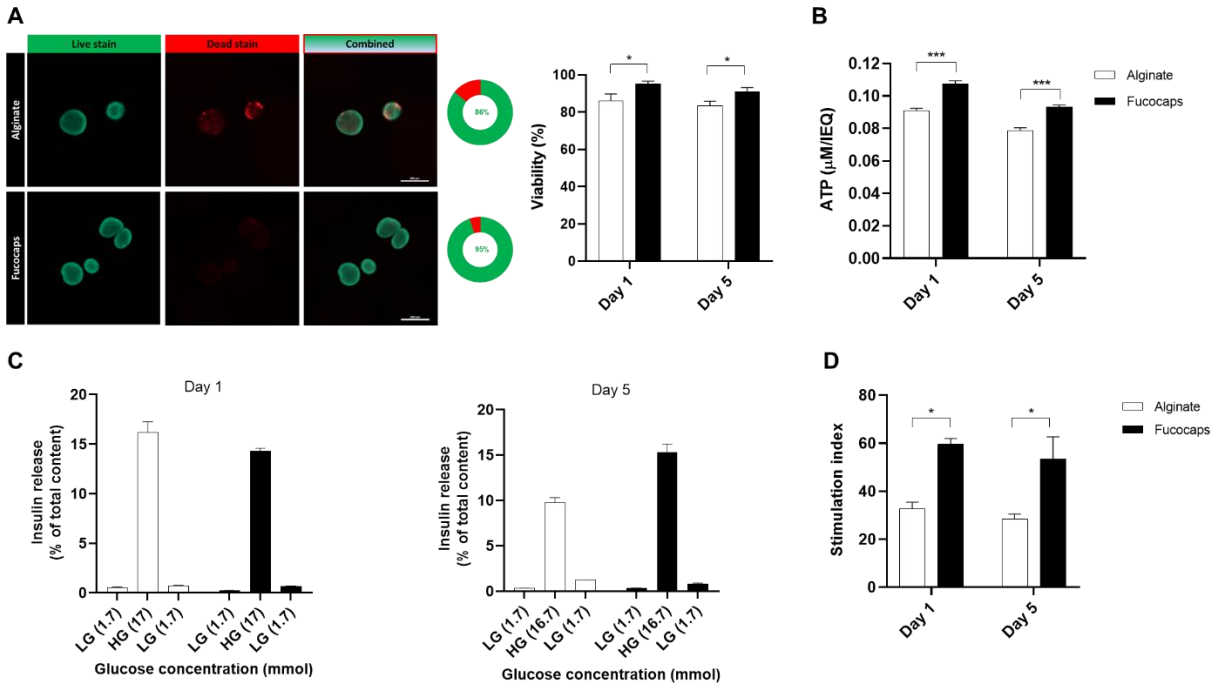


**Figure 6.** Encapsulation of rat islets in fucocaps: Representative viability images of rat islets encapsulated in alginate microcapsules and fucocaps at day 1 and 5 post-encapsulation (A) (Scale bar = 500 µm). Viability of islets in fucocaps was significantly higher on both days compared to those in alginate microcapsules. Values = mean ± SEM (n=25 islets); $^{*}p<0.05$ for viability when compared between alginate microcapsules and fucocaps both on days 1 and 5 (Student's t-test). Measurement of ATP levels, a marker of metabolically active cells both in alginate microcapsules and fucocaps (B). Values = mean ± SEM (n=5-6); $^{***}p<0.001$ for ATP levels when compared between alginate microcapsules and fucocaps both on days 1 and 5 (Student's t-test). Glucose stimulated insulin secretion (GSIS) on islets encapsulated in alginate microcapsules and fucocaps for days 1 and 5 post-encapsulation (C) and the corresponding stimulation indices (SI) (D). Values = mean ± SEM (n=3-4); $^{*}p<0.05$ for stimulation index (SI) when compared between alginate microcapsules and fucocaps both on days 1 and 5 (Student's t-test).

In order to verify whether the positive effect of FF fucoidan on insulin secretion in primary rat islets and pseudoislets is consistent between different species, a pilot study was carried out using neonatal porcine islets. The viability of neonatal porcine islets following

isolation dropped drastically during the 8 day culture period to produce mature beta cells. Despite the poor survival, encapsulation of neonatal porcine islets in fucocaps had higher viability compared to those in alginate microcapsules 48 hr post-encapsulation. Live/dead analysis demonstrated that viability of neonatal porcine islets in fucocaps (54.2 ± 0.7%) was significantly higher than those in alginate microcapsules (35.6 ± 1.5%) **(Figure 7B)**. As expected, there was a marginal but significant increase in the ATP levels in neonatal porcine islets encapsulated in fucocaps (0.042 ± 0.001 µM/IEQ) compared to those embedded in alginate capsules (0.04 ± 0.0002 µM/IEQ) (Figure 7C). As seen with rat islets, neonatal pig islets encapsulated in fucocaps functioned better with an increase in insulin release in response to high glucose with a significantly higher stimulation index (3.1 ± 0.4) compared to those in alginate microcapsules (1.4 ± 0.1) (Figures 7 D&E). Further, in the presence of forskolin (1 µM), which mimics the effect of GLP-1 by activating adenylate cyclase, thus raising intracellular cAMP and activating protein kinase A (PKA) [47], insulin secretion was increased 1.7 fold for neonatal islets encapsulated in fucocaps compared to those in alginate microcapsules (Figure 7D). These data suggest that encapsulation of islets in fucocaps proved beneficial in the short term by improving cell survival and function and the benefit observed could be translated across islets obtained from different species.

To our knowledge, this is the first study exploring the potential of fucoidan in the context of islet encapsulation. We used pseudoislets (from INS1E cells) as a model, and primary islets from rats and neonatal pigs for fucocaps microencapsulation and assessed cell viability and the insulin secretion. In all cases, and across the different species used in this study, we found that islets encapsulated within fucocaps demonstrated significantly improved viability and function. This beneficial effect may be attributed to the redox modulating property of fucoidan by induction of the antioxidant enzyme superoxide dismutase and glutathione activity (46). The encapsulation process by itself induces some degree of oxidative stress and we have shown that fucoidan can indeed significantly alleviate oxidative stress in encapsulated cells (Figure 5D).

Apart from its antioxidant properties, fucoidan has been shown to possess anti-diabetic properties by inducing insulin secretion, and reducing blood glucose levels, stimulating glucose uptake and improving insulin sensitivity [35–37]. We found that islets encapsulated within fucocaps responded better to changes in glucose with significantly higher insulin secretion and improved stimulation indices. Further, we have also shown that in high glucose conditions, fucoidan support the increase of insulin secretion in a dose- and time- dependent manner in comparison to controls (Figures 3 A&B). Fucoidan assisted insulin secretion may be attributed to an increase in cAMP concentrations which was shown to be significantly elevated in fucoidan treated insulin producing RIN-5F cells [37]. Elevation in cAMP levels has been shown to stimulate insulin exocytosis both in a $Ca^{2+}$ dependent and independent mechanisms and supports membrane depolarization by directly inhibiting KATP channels [38–40]. This is in concurrent with our findings in neonatal porcine islets where addition of forskolin, a cAMP stimulator, significantly increased insulin secretion in fucoidan containing microcapsules than those in alginate microcapsules suggesting a possible synergistic effect. However, the role of cAMP signalling pathways in mediating fucoidan induced insulin secretion needs to be fully elucidated.

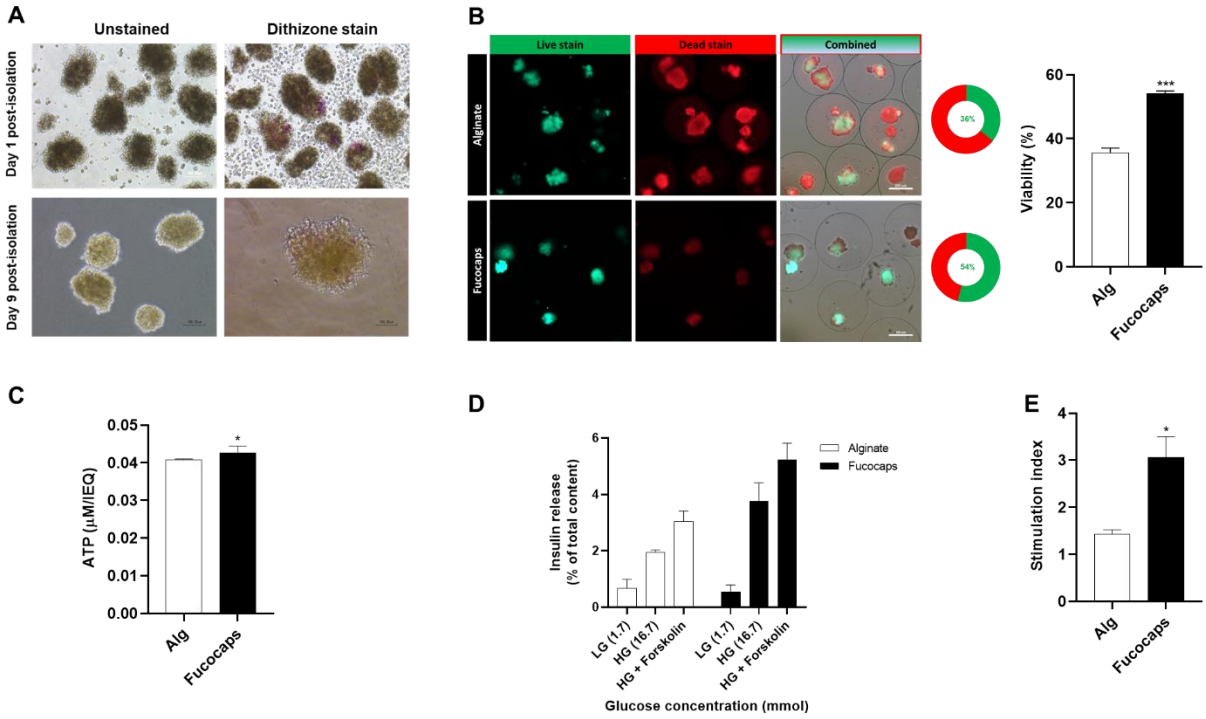


**Figure 7.** Encapsulation of neonatal pig islets in fucocaps: Representative optical microscopy images of neonatal pig islets immediately after isolation and day 9 post-isolation for maturation and their corresponding dithizone staining (A). Representative viability images of neonatal pig islets encapsulated in alginate microcapsules and fucocaps at day 2 post-encapsulation (B) (Scale bar = 500 µm). Viability of neonatal islets in fucocaps was significantly higher compared to those in alginate microcapsules. Values = mean ± SEM (n=25 neonatal islets); $^{***}p<0.001$ for viability when compared between alginate microcapsules and fucocaps (Student's t-test). Measurement of ATP levels, a marker of metabolically active cells both in alginate microcapsules and fucocaps (C). Values = mean ± SEM (n=3); $^{*}p<0.05$ for ATP levels when compared between alginate microcapsules and fucocaps (Student's t-test). Glucose stimulated insulin secretion (GSIS) on neonatal islets encapsulated in alginate microcapsules and fucocaps for day 2 post-encapsulation (D) and the corresponding stimulation index (SI) (E). Values = mean ± SEM (n=3-4); $^{*}p<0.05$ for stimulation index (SI) when compared between alginate microcapsules and fucocaps (Student's t-test).

**2.6 Effect of fucoidan on the cellular response pathway in pancreatic beta cells**

RNA-seq was carried out to understand the effect of fucoidan treatment on the varied cellular pathways in INS1E cells in the presence or absence of $H_2O_2$. Dimension reduction followed by CPM (counts per million) normalization demonstrated that controls and fucoidan treated biological duplicates are consistent and clustered together. $H_2O_2$ duplicates, however, exhibited very different transcriptional response with one sample clustering closer to the fucoidan group and another one being isolated in the dimension reduction (Figure S3A). The distance between the global pseudo-counts was confirmed in a heatmap generated from the mixOmics package (Figure S3B).

Differential gene expression analysis shows the presence of three main clusters **(Figure 8A)**. Cluster I is composed of genes that are lowly expressed in the controls but slightly expressed in the control samples. Among these genes, Fos and Fosb, have been described to be important for pancreatic β-cell proliferation[48]. Other genes such as Nr4a1 and Nr43 are associated with hormone stimulus and pancreatic β-cell proliferation. These results indicate that the fucoidan encapsulation might reduce β-cell proliferation.

Cluster II contain genes related to biosynthetic processes, and it is lowly on all the groups but slightly higher on the control group. Among these genes, regulators of cell cycle progression, such as Rgs2 and Atf3 are differentially regulated between the groups. These results reflect the induction of the $H_2O_2$-induced oxidative stress, in which cell cycle progression is highly affected.

Cluster III contains genes that are highly expressed in both groups but mostly more induced in the control group. These genes are involved in metabolic processes, such as response to reactive oxygen species and oxidative stress, as TXNIP and HIPK2 [49–51], and to carbohydrates, which might reflect the functionality of the pseudo-islets in processing sugars. These results indicate

that the pseudo-islets are still functional in the present culturing conditions, supporting the protective role of fucoidan on alleviating oxidative stress observed above.

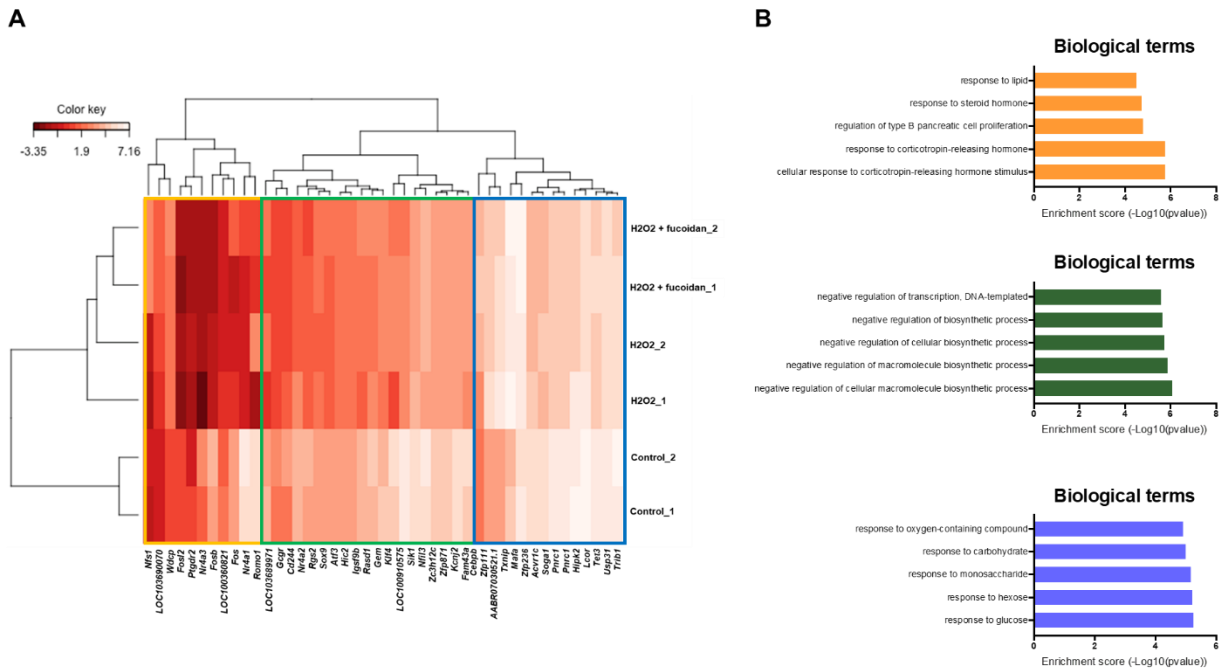


**Figure 8.** Gene expression clustering in INS1E cells. RNA-seq reads were aligned to the reference genome (rat rn6) using HISAT2 (version 0.1.6). Differential expression analyses were performed using EdgeR (version 3.12) with adjusted p-value < 0.05 as the cut-off. The expression data were grouped, and clustering analysis were performed on EdgeR (A). All Gene Ontology (GO) analyses were performed with ToppGene method (https://toppgene.cchmc.org/), and only Biological Process terms were kept for downstream analyses (B). Enrichment score was calculated from the as -log10 of the p-value.

## 3. Conclusion

To the best of our knowledge, the present study is the first to explore the potential of fucoidans for islet encapsulation. Our results showed that fucoidan from *Fucus vesiculosus* (FF) possess high fucose & sulfate content, exhibiting potent antioxidant capacity, significantly alleviated oxidative stress and enhanced the viability and function of INS1E pancreatic beta

cells. Interestingly, this protective and enriching effect of fucoidan was also observed on rat and pig pancreatic islets, as well as pseudo-islets formed from INS1E aggregates, when encapsulated in FF-containing alginate hydrogels (Fucogel). The derived microcapsules (Fucocaps) may also act as an immune barrier, which together with the provided redox-modulatory niche, results in an excellent biomaterial for enabling pancreatic islets transplantation therapies.

## 4. Experimental Section

*Fucoidans:* Commercially sourced fucoidan extracts isolated from two species of brown algae *Fucus vesiculosus* (*FF*) (Maritech Fucoidan, Marinova, batch # FVF2011527, Australia) and *Ascophyllum nodosum (FA)* (Ascophycient, Algues & Mer, batch # ASPHY16241, France), were purified using calcium acetate in order to reduce the natural heterogeneity and to get a more purified fucoidan fraction [24]. *Purification of fucoidans*: Both fucoidans (FF and FA) were purified using calcium acetate in order to reduce the natural heterogeneity and to get a more purified fucoidan fraction [24]. Briefly, both FA and FF extracts were dissolved (160 mg $ml^{-1}$) in 20mM calcium acetate, maintained and stirred for 4h at 55ºC with constant pH adjustment between 6.6 and 7.5; followed by overnight cooling at 4ºC and centrifugation for 15 min at 5000 rpm (Centrifuge 5810, Eppendorf, Germany). The resulting supernatant was dialyzed against water using 12-14 kDa cut-off dialysis membranes and freeze-dried (CryoDos-80, Telstar, Spain). The purified fractions of both FF and FA were used for subsequent characterization and encapsulation studies.

*Cell Culture:* a) *Rat β-cell (INS1E) culture:* INS1E β-cells, derived from rat insulinoma (AddexBio, USA) were cultured in RPMI 1640 (Gibco) supplemented with 5% (v/v) fetal bovine serum, 100 U $mL^{-1}$ penicillin and 100 mg $mL^{-1}$ streptomycin, 1mM sodium pyruvate and 10 mM HEPES. For cell expansion, 2-mercaptoethanol was added fresh to the culture to a

final concentration of 1 µl $mL^{-1}$ medium. Medium was refreshed every 2–3 days. *INS1E pseudoislet generation and culture:* Non-adherent agarose microwell chips were aseptically fabricated as described previously [52]. Briefly, microwell chips containing 1585 microwells with a diameter of 400 µm were fabricated by pouring a 3% (w/v) Ultrapure™ agarose (Invitrogen, Bleiswijk, The Netherlands) solution on negative molds of polydimethylsiloxane (PDMS) on 6 well plates. After agarose solidification, the molds were removed and chips transferred to 12 well plates, covered with PBS and stored at 4 °C until usage. Before cell seeding, the agarose chips were pre-incubated in culture medium overnight at 37 °C. For cell aggregate formation, single cells were resuspended in fresh medium and seeded onto agarose chips at a concentration of 800,000 cells per agarose chip. Immediately after seeding, agarose chips were briefly centrifuged for 1 min at 200 rpm to allow the cells to settle down in the microwells. The medium was refreshed every 1– 2 days. The pseudoislets were cultured up to 4 days after which they were harvested from the chips by upside down centrifugation (1 min @ 200rpm) and gently rinsing with medium and collected for further studies. The average pseudoislet diameter, was measured using light microscopy and ImageJ software [52]. *b) Primary rat islet isolation and culture:* Pancreatic islets from male Lewis rats (> 10 weeks) were isolated by ductal perfusion of collagenase (0.25 mg $ml^{-1}$) (Liberase TL Research grade, Roche Diagnostics GmbH, Germany) as described previously [53], followed by purification on Ficoll-Paque Plus (GE Healthcare, Merck, Netherlands) and separated from unwanted tissue debris using a stereomicroscope. The isolated islets were then cultured for 48 h in RPMI 1640 medium (Life Technologies, The Netherlands) supplemented with penicillin and streptomycin and 10% foetal bovine serum (FBS) in an incubator at 37°C supplied with 5% CO2 in air before being encapsulated. The purity of the islets was assessed by dithizone (Sigma-Aldrich, The Netherlands) staining and found to be >95%. All animal experimental procedures were approved by the Dutch ethical committee on animal experimentation (CCD) and the institute's Animal Care and Ethics Committee at Maastricht University (IVD). *c) Neonatal pig islet*

*isolation and culture:* All neonatal pig studies were conducted in accordance with the local ethical committee and with EU Directive 2010/63/EU for animal experiments. Piglets (Belgian landrace; 3-8 kg) were directly delivered to the islet isolation facility in Brussels. Neonatal pancreatic islet isolation was carried out as previously described [54]. Briefly, the harvested neonatal pig pancreas was cut into small pieces and transferred to a 50 ml tube. The pancreas was then washed twice with 30 ml of ice cold supplemented HBSS (10 mM Hepes, 100 U/mL Penicillin-streptomycin, 10 mM Glucose, 0.075% Amphotericin B, 0.25% BSA) with each time allowing the pancreatic tissue to sediment and removing the supernatant with debris and fatty tissues. After washing, collagenase V (Sigma-Aldrich, Belgium) (10 mg dissolved in 5 ml HBSS) was added, and the pancreas digested by manual shaking for 20 min at 37°C followed by addition of 40 ml of cold HBSS to stop the digestion process. Wash the pancreas 3 times by filtering the preparation on a sterile 500 μM mesh and suspend the filtered pancreas in supplemented HAM F10 medium. Neonatal pig islets (NPI) were cultured in HAM-F10 culture medium supplemented with 0.5% BSA, 50 μmol/L IBMX (3-isobutyl-1-methylxanthine), 10 mmol/L glucose, 2 mmol $L^{-1}$ glutamine, 10 mmol $L^{-1}$ nicotinamide, 1% penicillin, and 1% streptomycin during a period of 8 days to allow maturation of islet cells and removal of exocrine tissue as previously described before encapsulation [54].

*Cytotoxicity assay:* INS1E cells were seeded at $2 \times 10^4$ cells/well (96-well plate) for 24 hr at 37 °C in RPMI media (supplemented with 5% fetal bovine serum, 100 U $mL^{-1}$ penicillin and 100 mg $mL^{-1}$ streptomycin, 1mM sodium pyruvate and 10 mM HEPES). Thereafter, the media was removed, cells washed twice in serum free medium (SFM) before the addition of test samples FF and FA fucoidan in SFM in concentrations ranging from 0.15–100 mg $ml^{-1}$. Solutions of 5% PBS and 5% DMSO were prepared in SFM and used as negative and positive controls respectively. Test samples and controls were incubated with INS1E cells (100 μl/well in triplicates) for 24 hr, and cell viability measured by MTT (0.5 mg $ml^{-1}$) for the final 4 hr of

the culture period. The resultant insoluble formazan product was solubilised with DMSO and absorbance measured at 540 nm. Cell viability was expressed as a percentage of SFM incubated cells. A reduction of cell viability to < 70% as compared to SFM control medium alone was regarded as cytotoxic. The cytotoxicity assay was carried out as per ISO10993-5 "Biological evaluation of medical devices – Part 5: Tests for in vitro cytotoxicity".

*Physico-chemical characterization of fucoidans: a) NMR Spectroscopy:* The purified fucoidans (FF and FA) were characterized by $^{1}$HNMR spectroscopy. $^{1}$HNMR spectra of FA and FF fucoidans, dissolved (5 mg $ml^{-1}$) in deuterated water ($D_2O$), were recorded with a Burker Avance III 300 Hz at 90º impulses and 4s acquisition time. The resulting spectra were processed and analysed using Mnova v 14.1.0 software (Mestrelab research). b)*Quantification of carbohydrates*: Total carbohydrate analysis in purified FF and FA fucoidans was determined as described elsewhere [55,56]. Briefly, a pre-hydrolysis of FA, FF fucoidans was performed with 72% sulfuric acid for 3 h at room temperature followed by complete hydrolysis with sulfuric acid 1M at 100ºC for 2.5 h. Monosaccharides in the hydrolysates were reduced with sodium borohydride and acetylated by acetic anhydride using methylimidazole as a catalyst. The formed alditol acetate derivatives were analyzed by gas chromatography (GC) using a 30 m column DB-225 (J&W Scientific, Folsom, CA, USA), internal diameter, and film thickness of 0.25 and 0.15 mm, and a flame ionization detector (Perkin Elmer, Clarus 400). The hydrolysis of all samples was performed in duplicate, and each sample was injected twice. 2-Deoxyglucose (Alfagene, Portugal) was used as an internal standard. Uronic acids were quantified by a modification of the 3-phenylphenol colorimetric method [55,56]. A calibration curve was made with d-galacturonic acid. The hydrolysis and analysis of the samples was done in triplicates. *c) Quantification of sulphate content:* The quantification of sulphates in purified FF and FA fucoidans was performed as described previously [57]. Briefly, conditioning reagent composed by a mixing of 10 mL glycerol, 6 mL concentrated hydrochloric acid (HCl), 60 mL deionized

water, and 20 mL isopropyl alcohol, and sodium chloride was prepared (Sigma-Aldrich, Portugal). The conditioning reagent was stirred at room temperature overnight. Four replicate samples were prepared by weighing 10 mg $ml^{-1}$ of dried fucoidan into separate closed test tubes containing 2 mL 4 M hydrochloric acid (HCl). Samples were subjected to acid hydrolysis for 2 hours at 100ºC. A solution of potassium sulphate standards with concentrations ranging from 200-1000 µg $mL^{-1}$ was prepared. Barium chloride ($BaCl_2$) (0.3 g $ml^{-1}$) (Sigma -Aldrich, Portugal) was added, stirred for exactly 1 min and then left standing for 4-6 minutes to allow the barium sulphate ($BaSO_4$) precipitate to form. Absorbance was measured at 420 nm using a microplate reader (SynergyHT, Biotek Instruments, Portugal).

*Antioxidant properties of fucoidans: a) Total antioxidant capacity assay:* Measurement of the total non-enzymatic antioxidant capacity of biological samples provide us with some insight of their ability to counteract oxidative stress-induced damage in cells. The total antioxidant capacity of purified FF and FA fucoidans expressed as the Trolox equivalent antioxidant capacity (TEAC) was determined using a Total Antioxidant Capacity Assay kit (Sigma-Aldrich, The Netherlands) according to the manufacturer's instructions. Briefly, small molecule anti-oxidants convert $Cu^{2+}$ ions to $Cu^{+}$, after which reduced $Cu^{+}$ ions chelate with a colorimetric probe resulting in a absorbance peak at 570 nm. The height of the absorbance peak is proportional to the total antioxidant capacity expressed as TEAC. Trolox, a water-soluble vitamin E analogue is used as an antioxidant standard in all conditions. Purified FF and FA fucoidans samples ranging from 4-20 nmoles/well were prepared in a protein mask solution (to prevent the $Cu^{2+}$ reduction by protein) followed by addition of a $Cu^{2+}$ working solution and incubation for 90 min at room temperature after which the absorbance at 570 nm was measured using a CLARIOstar microplate reader. The amount of antioxidant in fucoidans can subsequently be determined using the Trolox reference curve and is expressed as TEAC; nmole, from which the antioxidant concentration in Trolox equivalents (nmole $µl^{-1}$), can be calculated.

b) *Free radical scavenging assay:* The scavenging activity of FF and FA fucoidans against free radicals was measured using the DPPH (2, 2-diphenyl-1-picrylhydrazyl) (Sigma-Aldrich, The Netherlands) assay. Briefly, Ascorbic acid, FF and FA fucoidans were dissolved in water (1 mg $ml^{-1}$) and 200 µl of the above solution added to each well of a 96-well plate. The samples were then serially diluted in water for varied concentrations ranging from 1000 µg $ml^{-1}$ to 1.56 µg $ml^{-1}$. Freshly prepared DPPH solution (0.2 mM) in methanol was prepared and 100 µl of the resulting solution was mixed thoroughly with 100 µl of FF and FA fucoidans of varied concentrations. The plate was then covered with aluminium foil (to protect DPPH radical degradation by light) and the reaction mixture was incubated for 1 h at room temperature. Absorbance was then measured at 515 nm using microplate reader (Clariostar). Ascorbic acid dissolved in water (1 mg $ml^{-1}$) was used as a standard and solvent alone (methanol: water; 1:1) was used as a blank. DPPH solution alone (100 µl) mixed with water (100 µl) served as control. The percentage of radical scavenging was obtained using the equation:

$$\%\ DPPH_{scavenging} = 100 \times \left[\left(Abs_{sample} + DPPH\right) - \left(Abs_{Sample\ blank}\right)\right] / \left[\left(Abs_{DPPH}\right) - \left(Abs_{solvent}\right)\right]$$

and the $IC_{50}$ values calculated using the GraphPrism software (GraphPad Software, USA). Ascorbic acid was used a positive control in this study. *Intracellular oxidative stress assay:* Intracellular oxidative stress levels were determined using the 2´,7´-dichlorodihydrofluorescein-diacetate (DCFH-DA) (Sigma-Aldrich, The Netherlands) [33]. A stock solution of 10 mM DCFH-DA was made in DMSO; dilutions were made in exposure medium comprising RPMI supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 200 g $L^{-1}$ glucose and 200 mM L-glutamine. For direct measurements, INS1E cells were seeded at a density of 140,000 cells/well in a black 96 well imaging plate with clear bottom and cultured overnight. Cells were then incubated for 45 min with 20 µM DCFH-DA at 37°C and 5% $CO_2$ after which cells were washed using PBS. After DCFH-DA exposure, cells were incubated with

fucoidan containing media (FA & FF; 10 mg $ml^{-1}$) in the presence or absence of 2 mM $H_2O_2$. Fluorescence was measured at 5 min interval over a period of 60 min in a microplate reader (CLARIOstar) at an excitation wavelength of λ = 485 nm, and emission wavelength at λ = 538 nm. The area under the curve was considered the total oxidative stress experienced by cells, and was normalized to the number of viable cells in each sample, and compared to reference sample of non-treated cells cultured for 24 h in tissue culture polysterene. Viability in each condition was calculated as percentage relative to non-treated controls.

*Effect of fucoidan on insulin secretion:* To determine the direct effects of fucoidan on insulin secretion, INS1E cells were seeded into 24-well plates at a density of 200,000 cells/well in supplemented RPMI 1640 medium and incubated at 37°C for 24 h. The cells were then washed in PBS and exposed to low glucose (2.8 mM), high glucose (20 mM), high glucose in the presence of varied FF fucoidan concentrations (0.01, 0.1, 0.5, 1, 5, and 10 mg $ml^{-1}$) for 3 h and high glucose in the presence of 5 mg $ml^{-1}$ FF fucoidan for varied time points (6, 12 and 24 h). Aliquots of the media were removed from each of the wells and centrifuged (1,175 x g, for 5 min at 4ºC) to remove the cells and stored at -20ºC. The cells were exposed to 600 µl of acid ethanol, incubated overnight at 4ºC, and the supernatant collected the following day for measuring insulin content. The concentration of insulin in the supernatants was determined using a rat insulin ELISA kit (Mercodia, Uppsala, Sweden).

*Characterization of fucoidan alginate hydrogels and fucoidan alginate microcapsules: a) Antioxidant capacity of fucoidan containing alginate hydrogels (Fucogels):* The total antioxidant capacity of ultrapure sodium alginate (> 60% G sodium alginate Pronova™, UPMVG, Novamatrix, FMC Biopolymer, Norway) hydrogel and ultrapure sodium alginate hydrogel with fucoidan was determined using the Total Antioxidant Capacity Assay kit (Sigma-Aldrich). For this study, 2.5% w/v of alginate solution was made in protein mask solution. For the alginate/fucoidan hydrogel (Fucogel), 0.5% w/v purified FF fucoidan was mixed with 2%

w/v ultrapure sodium alginate to obtain a 2.5% w/v Fucogel in protein mask solution. Both alginate and fucogel were diluted further in protein mask solution to provide final concentrations of 0.5%, 1%, 1.5%, 2% and 2.5% w/v and from each sample the TEAC and antioxidant concentration was determined. *b) Rheology and viscoelastic properties of fucogel:* Rheology experiments were performed with a Discovery HR-2 hybrid rheometer (TA instruments, The Netherlands) with a 8 mm flat plate geometry (TA instruments, serial number 109413). A total of 175 µl hydrogel precursor solution was casted in a 12 mm silicon mold and crosslinked in an excess of 20 mM $BaCl_2$ for 5 minutes. A solvent trap was placed around the sample and filled with distilled water to prevent solvent evaporation from the gel. Hydrogels were preconditioned with a time sweep hold with a 1% strain at an angular frequency of 10 rad/s. Initially, hydrogels were subjected to a frequency sweep with a strain of 1% and frequencies ranging between 1-500 rad $s^{-1}$ to determine a suitable frequency for the strain sweep. Next, hydrogels were exposed to a strain sweep with strains ranging between 0.01-10% and an angular frequency of 10 rad $s^{-1}$ to determine the linear viscoelastic region of the hydrogels. The storage modulus (G') was monitored during the strain sweep and used to calculate the hydrogel mesh size ($D_{mesh}$) based on the formula:

$$D_{mesh} = \left(\frac{6\,RT}{\pi N_{av} G'}\right)^{\frac{1}{3}}$$

The classical theory of rubber elasticity relates the shear modulus (G') to the mesh size $r_{mesh}$ (formula 1), where R is the gas constant, T the absolute temperature and Nav is Avogadro's number. This allows estimation of the mesh size of a hydrogel based on its shear modulus [58,59]. The mesh size ($D_{mesh}$) represents the diameter of a hard sphere that fills the void in between hydrogel network strands. c) *Generation of alginate and alginate/fucoidan microcapsules (Fucocaps):* The formation of both alginate and alginate/fucoidan (Fucocaps) microcapsules were carried out using an air-driven droplet generator (Nisco D-0038, Zurich, Switzeland,

Nozzle from Biored, California, USA) as described previously [53]. Briefly, for microcapsule formation, 2.2% w/v sodium alginate and 2.5% w/v fucogel polymer solutions were injected through the droplet generator with a steady airflow rate of 5 L/min. The microcapsules were collected on a sterile 145 mm petri dish containing 50 ml $BaCl_2$ (20 mM) working as a gelling solution, which was placed at a distance of 16.5 cm from the tip of the encapsulation device to bath. The microcapsules formed were then incubated in $BaCl_2$ solution for 2 min, followed by five-time washings with Dulbeco´s phosphate buffered saline (DPBS) (Sigma-Aldrich) to remove excess $BaCl_2$. After repeated washings, the microcapsules were stored in DPBS and microcapsule size determined by optical microscopy (Nikon TiS microscope, Nikon, The Netherlands). d)*Toluidine blue staining:* The presence of fucoidans in microcapsules was visualized using a toluidine blue O (TBO) staining. Briefly, alginate microcapsules and fucocaps were randomly collected and immersed in 1 ml of TBO solution (0.1 M HCl, 20 mg NaCl, and 4 mg toluidine blue O chloride) for 30 min at room temperature. The microcapsules were then washed in DPBS to remove excess TBO solution and incubated further for 24 h. After 24 h incubation, the microcapsules were washed again, stored in DPBS and aliquots of microcapsules were randomly chosen at different time points for imaging using optical microscopy (Nikon TiS microscope).

*Encapsulation: a) Encapsulation of insulin producing cells:* Before the microencapsulation procedure, INS1E pseudoislets, primary rat islets and neonatal pig islets were pooled and mixed thoroughly with either 2.2% w/v ultrapure sodium alginate, or a 2.5% w/v ultrapure alginate fucoidan solution in a 1:8 ratio (cells: alginate) in a 50 ml falcon tube. Subsequently, the alginate cell mixture was then extruded through the nozzle of the droplet generator with an airflow of 5 L $min^{-1}$ leading to the formation of uniform droplets. The droplets were collected in a sterile 145 mm petri dish containing 50 ml $BaCl_2$ (20 mM) gelling solution to crosslink the alginate molecules after which cell containing microcapsules were formed. The microcapsules were left

to incubate in the $BaCl_2$ solution for 2 min after which they were collected, and washed five times with DPBS to remove any excess $BaCl_2$. After washing, the microcapsules were allowed to settle down, and the remaining supernatant was discarded. The microencapsulated cells and islets were then cultured for a 24 h in appropriate media before being used for further *in vitro* studies. b) *Encapsulation of INS1E reporter cells:* To explore the potential of fucoidan to alleviate oxidative stress post-encapsulation, we used a genetically encoded redox probe mt-roGFP2-Orp1 transduced INS1E beta cells (passage 93). Stably expressing mito-roGFP2-Orp1 INS1E cells were obtained from Leticia Prates Roma (Saarland University). INS1E cells were retrovirally transduced with pLPCX mito roGFP2-Orp1 as previously described for H1975 cells [60,61]. The stably transduced mito-roGFP2-Orp1 INS1E cells were cultured in RPMI (Gibco 61870) supplemented with 5% (v/v) fetal bovine serum, 10 mM HEPES, 1 mM sodium pyruvate, 100 U/ mL penicillin, 100 mg $mL^{-1}$ streptomycin and 0.05 mM 2-mercaptoethanol at 5% CO2 at 37°C. INS1E reporter cells (1 x $10^6$) were encapsulated in alginate and fucocaps as described above and cultured for 24 hr. The following day the encapsulated cells were washed in PBS and exposed to $H_2O_2$ (1 mM) for 1 hr to induce oxidative stress. To induce reduction and oxidation DTT (5 mM) and Diamide (0.5 mM) were added respectively as controls and the OxDmtroGFP2 was calculated as previously described [62].

*Cell viability and ATP measurements:* The viability of non-encapsulated and encapsulated cells was assessed using the fluorescent dyes Calcein AM (for live cells) and Ethidium homodimer-1 (for dead cells) (Thermo Fisher Scientific, The Netherlands). Briefly, aliquots of non-encapsulated and encapsulated cells were randomly collected from the tissue culture dishes with a Gilson P 1000 µl pipette under sterile conditions. They were washed twice by suspending them in 0.5 ml PBS followed by careful removal of the supernatant and discarded. Subsequently, a solution of 4 ml of PBS and 2.5 µl of calcein AM and 5 µl of ethidium homodimer-1 were prepared and 1 ml of this solution added to each sample and incubated for

30 mim in darkness. The non-encapsulated and encapsulated cells were then visualized under a fluorescence microscope (Nikon Eclipse E600), and the percentage of green (live cells) to red (dead cells) was estimated in 25 microcapsules. For ATP measurements, aliquots of encapsulated cells were washed twice by suspending them in 0.5 ml PBS, discarding the supernatant and suspending again in 100 µl of PBS followed by addition of 100 µl of CellTiter-Glo® 3D Reagent (Promega). The contents were mixed vigorously for 5 min to induce cell lysis and the plates allowed to incubate at room temperature for a further 25 min to stabilize the luminescent signal. After 30 min of incubation, luminescence was measured on a microplate reader (CLARIOstar) and the amount of ATP measured which signals the presence of metabolically active cells.

*Glucose stimulated insulin secretion (GSIS) assay:* For GSIS assay, different concentrations of exposure solutions were made in Kreb's buffer containing 0.2% bovine serum albumin. Accordingly, for non-encapsulated rat islets, encapsulated rat and neonatal pig islets low glucose (LG) solution (1.7 mM) and high glucose (HG) solution (16.7 mM) were prepared and sterile filtered. For encapsulated pseudoislet derived from INS1E cells low and high glucose exposure solutions of 2.8 mM and 20 mM were prepared respectively. On the day of GSIS, aliquots of non-encapsulated and encapsulated islet/pseudoislet were handpicked and placed on inserts in 24 wells and washed thrice in low glucose solution (1.7 mM or 2.8 mM) followed by equilibration for 1.5 h at 37°C in low glucose solution. After equilibration, the cells were sequentially exposed to low (1.7 mM or 2.8 mM), high (16.7 mM or 20 mM) and low (1.7 mM or 2.8 mM) glucose concentrations with a 1.5 h incubation at 37°C for each exposure condition. Non-encapsulated rat islets and encapsulated neonatal pig islets were exposed to only low (1.7 mM) and high (16.7 mM) glucose concentrations for 1 hr. After each incubation, the supernatant was collected and stored for insulin measurements. After the final incubation step, the encapsulated cells were washed in PBS followed by addition of cold acid ethanol and

vortexed vigorously to enhance cell lysis. The extracts were kept at 4°C overnight and the supernatant collected the following day for measuring insulin content. Insulin secretion was quantified by using a rat and pig insulin ELISA kit (Mercodia, Uppsala, Sweden) according to the manufacturer´s instructions. Results were presented as low–high–low profiles normalized to total insulin content and as stimulation index, which is the ratio of insulin secretion between the high and low glucose conditions.

*RNA-sequencing: a) RNA isolation:*INS1E cells (1 x $10^6$/well) were seeded onto 6 well plates and the cells exposed to $H_2O_2$ (1 mM) for 1 hr in the presence or absence of fucoidan (5 mg $ml^{-1}$). Following incubation, the cells are washed in PBS, lysed and RNA isolation performed according to the RNeasy Mini Kit (Qiagen, Netherlands) and quantified using the BioDrop (Biochrom, United Kingdom). *b) RNA-seq sample preparation:* The KAPA Stranded mRNA-Seq Kit, with KAPA mRNA Capture Beads (Roche, cat# 07962207001) protocol was performed on 500ng of initial RNA from bulk material. RNA size selection was performed for 300pb fragments. RNA-seq libraries were amplified with 8 cycles. The purified libraries were quantified using Qubit (ThermoFisher) and the fragment distribution analyzed using TapeStation (Agilent). Complete libraries were sequenced (paired-end) on Illumina HiSeqTM4000, with 151bp from each end. *c) Transcriptomic analysis:* RNA-seq reads were aligned to the reference genome (rat rn6) using HISAT2 (version 0.1.6) with default parameters. Read counts representing gene expression levels were calculated with feature Counts (version 2.0.0) using default parameters. Only genes with counts above zero were retained for the downstream analyses. Differential expression analyses were performed using EdgeR (version 3.12) with adjusted p-value < 0.05 as the cut-off. Factor normalization was done using the CPM method. Dimension reduction and clustering analysis were performed on EdgeR. All Gene Ontology (GO) analyses were performed with ToppGene method (https://toppgene.cchmc.org/), and only Biological Process terms were kept for downstream

analyses. Enrichment score was calculated from the as -log10 of the p-value. Plots were created using GraphPad Prism7.

*Statistical analysis:* All data are presented as mean ± standard error of mean (SEM). Differences between two groups were analysed by the two-tailed Student's t-test and more than two groups by one-way analysis of variance (ANOVA) with post-hoc Multiple-Comparison test using GraphPad Prism software (GraphPad Software, USA). Significant differences among data groups were assigned when $p < 0.05$.

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The present study is the first to explore the potential of fucoidan, marine sulfated polysaccharide, for pancreatic islet encapsulation. Fucoidan has high fucose contents with sulfate groups, demonstrating potent antioxidant properties significantly alleviating oxidative stress and enhancing the viability and function of encapsulated beta cells. Fucoidan based hydrogel was thus assessed as biomaterial for bioengineered immunoprotective beta cell replacement systems.

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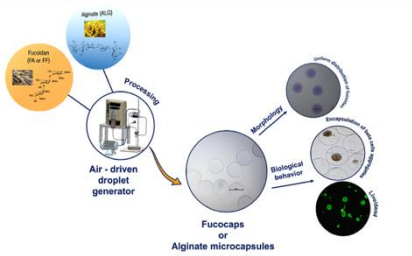
**Fucoidan hydrogels significantly alleviate oxidative stress and enhance the endocrine function of encapsulated beta cells**



# Supporting Information

**Fucoidan hydrogels significantly alleviate oxidative stress and enhance the endocrine function of encapsulated beta cells**

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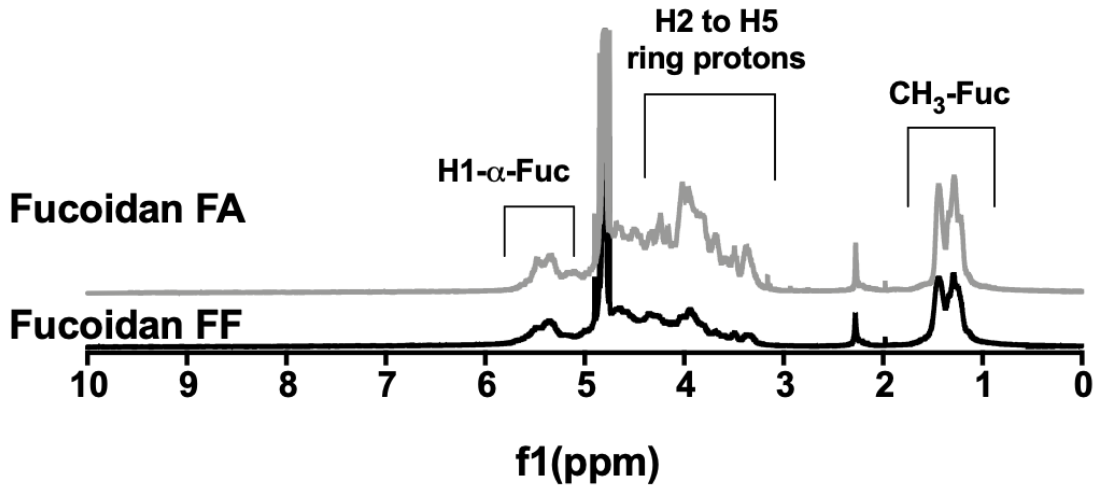


**Figure S1.** NMR spectra of purified fucoidans: $^{1}$HNMR of purified FF & FA fucoidans, spectra show different peaks δ = 1.20-1.30 and 1.95-2.26 ppm corresponding to methyl groups of fucose and L-fucopyranose; δ = 3.35-4.55 ppm confirmed the presence of different types of fucose groups with changes in glycosidic linkage positions and monosaccharides patterns, δ = 5.29-5.40 ppm corresponding to anomeric protons of L-fucopyranosyl unit.

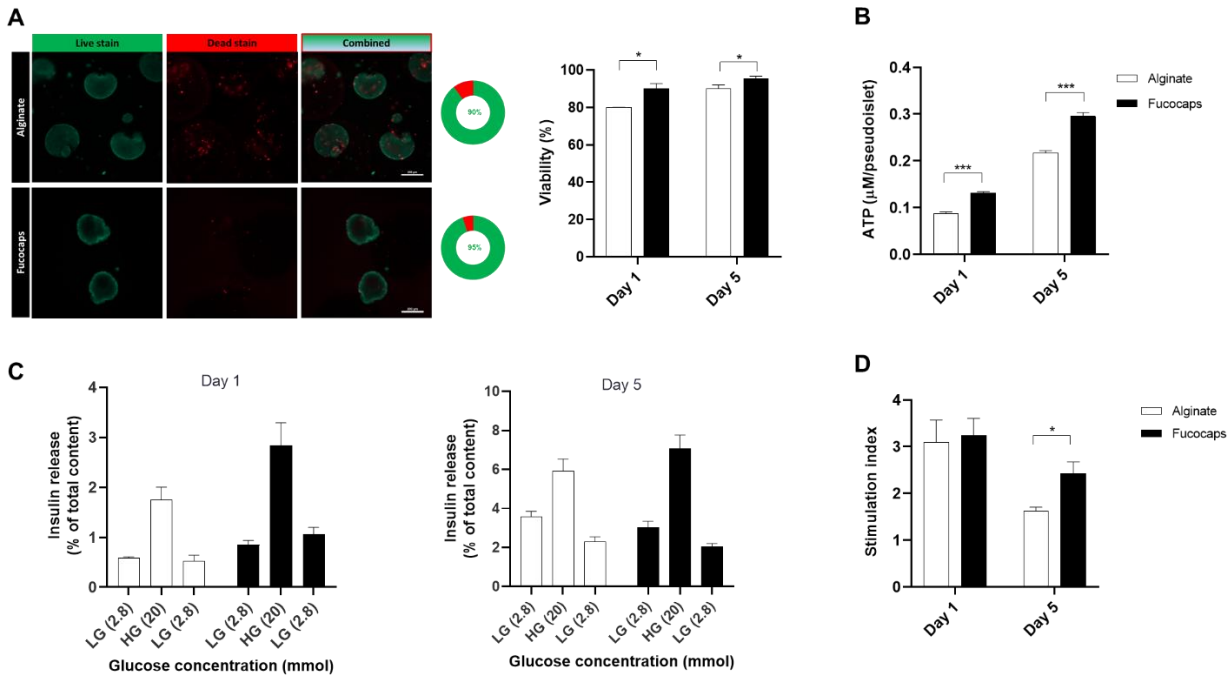


**Figure S2.** Encapsulation of pseudoislets in fucocaps: Representative viability images of pseudoislets encapsulated in alginate microcapsules and fucocaps at day 1 and 5 post-encapsulation (A) (Scale bar = 500 µm). Viability of islets in fucocaps was significantly higher on both days compared to those in alginate microcapsules. Values = mean ± SEM (n=25 pseudoislets); $^{*}p<0.05$ for viability when compared between alginate microcapsules and fucocaps both on days 1 and 5 (Student's t-test). Measurement of ATP levels, a marker of metabolically active cells both in alginate microcapsules and fucocaps (B). Values = mean ± SEM (n=5-6); $^{***}p<0.001$ for ATP levels when compared between alginate microcapsules and fucocaps both on days 1 and 5 (Student's t-test). Glucose stimulated insulin secretion (GSIS) on pseudoislets encapsulated in alginate microcapsules and fucocaps for days 1 and 5 post-encapsulation (C) and the corresponding stimulation indices (SI) (D). Values = mean ± SEM (n=3-4); $^{*}p<0.05$ for stimulation index (SI) when compared between alginate microcapsules and fucocaps on day 5 (Student's t-test).

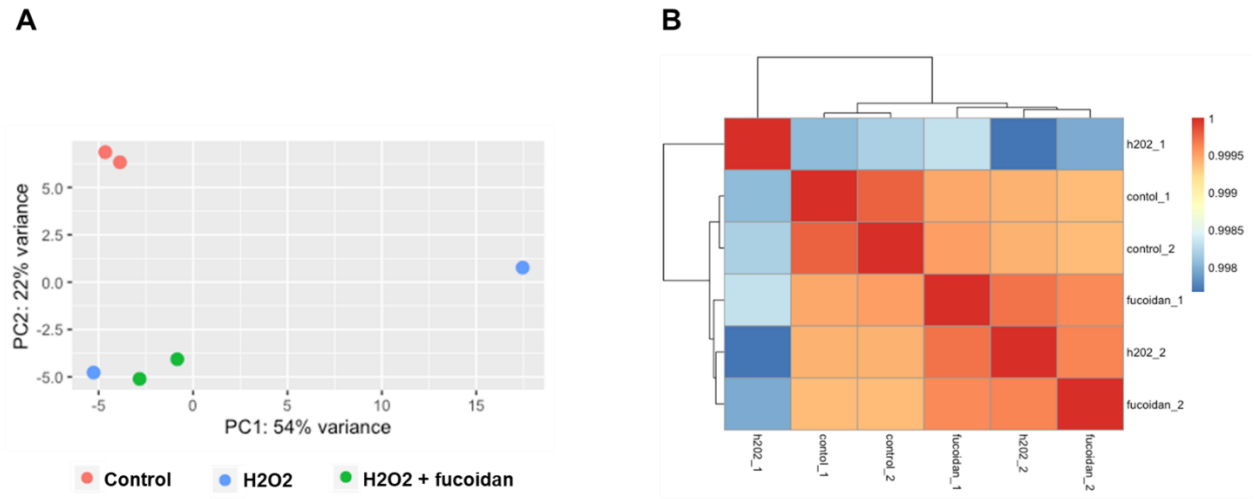


**Figure S3.** Differential expression analysis of the RNA-seq data: Dimension reduction followed by CPM (counts per million) normalization demonstrated that controls and fucoidan treated biological duplicates are consistent and clustered together (A). $H_2O_2$ duplicates did not cluster together and exhibited very different transcriptional response with one sample clustering closer to the fucoidan group and another one being isolated in the dimension reduction (A). Heatmap generated using the mixOmics package confirm the distance between the global pseudo-counts (B).