Hypolipidemic, antiobesity and cardioprotective effects of fermented protein hydrolysates from sardinelle (*Sardinella aurita*) in high-fat and fructose diet fed Wistar rats

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ABSTRACT

The present study aims to evaluate the antiobesity, hypolipidemic and cardioprotective effects of fermented sardinelle (*Sardinella aurita*) protein hydrolysates (FSPHs) produced with two proteolytic bacteria, *Bacillus subtilis* A26 (FSPH-A26) and *Bacillus amyloliquefaciens* An6 (FSPH-An6). Wistar rats were fed during 10 weeks a standard laboratory diet, a high caloric diet (HCD) and a HCD coupled with the oral administration of sardinelle meat flour (SMF) or FSPHs. HCD caused hyperlipidemia and increased body weight (BW). Interestingly, the daily oral administration of FSPHs or SMF reduced the total cholesterol (TC), triglycerides (TG) and low-density lipoprotein cholesterol (LDL-c) serum levels, and increased the level of high-density lipoprotein cholesterol (HDL-c). FSPHs also lowered hepatic TC and TG content and decreased the pancreatic lipase activity. Further, the administration of FSPHs or SMF decreased the BW gain, the food intake and the relative epididymal adipose tissue weight. FSPHs exhibited a potent cardioprotective effect against heart attack, which was demonstrated by returning atherogenic indexes to their normal levels and the conservation of standard histological structure of the heart and aorta. The overall results indicate that FSPHs contained bioactive peptides which significantly attenuated hyperlipidemia, and might reduce the risk of cardiovascular disease (CVD) in rats fed HCD.

1. Introduction

Hyperlipidemia is an elevation of lipids, including fats, fatty acids, cholesterol, cholesterol esters, phospholipids, and triglycerides, in the blood stream [1]. It is the major cause of atherosclerosis and atherosclerosis-associated conditions such as coronary heart disease, ischemic cerebrovascular disease, and peripheral vascular disease. A causal relationship between the elevated plasma lipids and the development of atherosclerotic plaques has been well established. The influence of the hypercaloric diet on the development of the cardiovascular diseases is reported in several studies [2,3]. In fact, the annual sudden cardiac death rate was nearly 40 times higher in obese people than in non-obese population [4]. The consumption of high-fat / fructose diets is correlated with high rates of overweight, central obesity and metabolic syndrome [5,6]. Zou et al. [7] reported that continued consumption of high-fat diets, especially which are enriched with cholesterol and animal fats, represents the major cause of hyperlipidemia, hepatic lipid accumulation, lipid peroxidation and hepatotoxicity. In addition, further studies have reported that the high fructose-rich diets are usually associated with cardiac alterations by inducing metabolic disorders characterized by the excessive body weight gain, hyperlipidemia and even atherosclerosis [8].

Several works have reported the importance of dietary proteins and protein hydrolysates in the regulation of cholesterol metabolism [9,10,11]. To date, hypocholesterolemic properties have been reported for soy [12] and whey [13]. Soy protein hydrolysates were found to exhibit a higher hypocholesterolemic activity than the undigested proteins [14]. Wergedahl et al. [15] reported that treatment of high fat fed Zucker rat with salamon protein hydrolysate reduced serum and liver cholesterol levels. Ben Khaled et al. [9] observed that the supplementation of sardinelle protein hydrolysate in the high cholesterol diet of wistar rat could decrease the levels of serum TG, TC and the LDL-c and increased the HDL-c content. A number of studies have focused on other beneficial effects of fish proteins and bioactive peptides derived from fish protein hydrolysates. In addition, antihypertensive and antioxidant effects [16], immunomodulating properties [17], and reparative properties in the intestine [18], have been reported.

The sardinelle (*Sardinella aurita*) is common in Mediterranean Sea, Atlantic, east on all of the west African coast and the west of Cape Cod in Argentina, the western Pacific from Japan to the Philippines [19]. Its first maturity is at 14 cm length. It is relatively important in the fishcatches of Tunisia and is utilised for human consumption. In Tunisia, sardinelle (*Sardinella aurita*) catches were about 14,200 tons in 2009. Our previous works showed that

sardinelle protein hydrolysates obtained by fermentation using *B. subtilis* A26 possess interesting biological activities such as antioxidant, antibacterial and anti-ACE.

In the present study, we investigated the potential of sardinelle meat flour (SMF) and protein hydrolysates obtained through fermentation by *Bacillus subtilis* A26 (FSPH-A26) and *Bacillus amyloliquefaciens* An6 (FSPH-An6) to lower serum lipids levels in rats fed with high fat and fructose diet. Their antiobesity effect and their beneficial role in reducing the atherogenic indexes and protecting the heart were also studied.

2. Materials and methods

2.1. Materials

Sardinelle (*Sardinella aurita*) was freshly purchased from the fish market of Sfax City, Tunisia. The samples were packed in polyethylene bags and transported to the laboratory within 30 min. After washing fishes with tap water, the muscles were separated and then rinsed with cold distilled water. They were used immediately for protein hydrolysates production or stored in sealed plastic bags at -20 °C until they were used, less than 1 week later.

2.2. Sardinelle meat flour preparation

Raw muscle from sardinelle (500 g) in 1000 ml of distilled water was cooked for 20 min at 100 °C. The bones were removed from cooked fish and fillets were collected and dried in an oven at 80 °C for 18 h. The dried fish preparation was minced to obtain fine powder.

2.3. Production of fermented sardinelle protein hydrolysates

B. subtilis A26 (CTM 50700) [20] and *B. amyloliquefaciens* An6 [21], known to produce several proteolytic enzymes, were used to produce protein hydrolysates through fermentation of sardinelle proteins. For the production of protein hydrolysates, the strains were grown in medium containing only powdered sardinelle meat (30 g/l; pH 8.0) as carbon and nitrogen sources. The media was autoclaved at 121 °C for 20 min. Cultivations were conducted in 1000 ml Erlenmeyer flasks containing 100 ml of culture media. Incubations were carried out in a shaking incubator (Technico Ltd, Chennai, India) with agitation at 200 rpm for 24 h at 37 °C. The growth of the microorganisms was estimated by the determination of colony forming units per milliliter. Cultures were then centrifuged at 8500×g for 30 min at 4 °C. The resulted cell-free supernatants, containing FSPHs, were freeze dried using freeze dryer (Bioblock Scientific

Christ ALPHA 1-2, IllKrich-Cedex, France) to obtain powders and stored at −20 °C for further use.

2.4. Reversed-phase high performance liquid chromatography (RP-HPLC)

The hydrophobicity of peptides from FSPHs was studied by reversed phase HPLC using an Agilent 1100 HPLC system (Agilent Technologies, CA, USA). The column used in this experiment was a Symmetry C18 (4.6 × 250 mm, 5 μm) from Waters Co. (Milford, MA, USA). Solvent A was TFA in bidistilled water (0.1% v/v) and solvent B contained TFA (0.085% v/v) in acetonitrile (ACN): bidistilled water (60:40 v/v). Both mobile phases A and B were filtered through a 0.45 μm nylon membrane filter and degassed prior to any analytical run. Peptides were first eluted with 100% solvent A for 2 min, followed by a linear gradient from 0% to 40% of solvent B during 50 min, then from 40% to 100% of solvent B during 60 min, at a flow rate of 1 ml/min. The separation was monitored at a wavelength of 214 nm.

2.5. Maldi-ToF mass spectrometry analysis

The analysis was done in a 5800 MALDI-ToF/ToF instrument (AB Sciex) in positive reflectron mode (3000 shots every position) in a range from 150 to 2000 Da; the laser intensity was manually adjusted to maximize the S/N ratio. Plate model and acquisition method were calibrated by AB SCIEX calibration mixture (des-Arg1-Bradykinin at 1 fmol/μl; Angiotensin I at 2 fmol/μl; Glu1-Fibrinopeptide B at 1.3 fmol/μl; ACTH (1–17 clip) at 2 fmol/μl; ACTH (18–39 clip) at 5 fmol/μl; and ACTH (7–38 clip) at 3 fmol/μl) in 13 positions. Dried hydrolysates were dissolved in 5% ACN containing 0.1% TFA, and 1 μl of every sample was directly spotted on 10 positions in the MALDI plate and allowed to air dry. Once dried, 0.5 μl ofmatrix solution (5 mg/ml of α-Cyano-4-hydroxycinnamic acid (CHCA) in 0.1% TFA–ACN/H_2O (7:3, v/v)) was spotted.
The analysis of data was done by using mMass software (http://www.mmass.org/).

2.6. Animals and experimental design

Male Wistar rats weighing about 110–150 g were purchased from the breeding center of the Central Pharmacy of Tunis (SIPHAT, Tunisia). Animals were kept in an environmentally controlled breeding room (temperature: 25±2 °C, relative humidity: 60 ± 5% and a 12 h dark /light cycle) in the laboratory of the Faculty of Sciences of Sfax, Tunisia. They were allowed free access to tap water and alimentation during the experimental period. Laboratory animal

handling and experimental procedures were performed according to the guidelines of the Tunisian Ethical Committee for the care and use of laboratory animals. Before treatments, rats were fed a standard diet for a 1-week period of acclimatization after arrival.

The rats were divided into six groups (n=6 rats each). Group 1, which served as control group (CD), was fed a standard diet supplied by Society of Animals Nutrition, Sfax, Tunisia. The characteristic of the standard diet was illustrated in the Table 1. Group 2 was fed a hypercaloric diet (HCD) during ten weeks, which was prepared by adding 10 % sheep fat, 5 % fructose and 0.1 % cholic acid to standard diet, to induce obesity and hyperlipidemia. The cholic acid is a bile acid involved to facilitate the formation of micelles, which promotes processing of dietary fat. Group 3 were rats fed HCD and received orally fluvastatin (20 mg/kg, BW/day). Fluvastatin (Fluv) (trade names Lescol, Canef and Vastin) is a member of the drug class of statins, used to treat hypercholesterolemia and to prevent from cardiovascular disease. Groups 4, 5 and 6 were fed a HCD and received daily, by oral gastric gavage 400 mg/ kg, BW of SMF, FSPH-A26 and FSPH-An6, respectively. Food intakes (FI) of the rats were recorded daily, and their body weights were monitored twice a week throughout the experiment.

At the end of the exprimental period (10 weeks), wistar rats were weighted and then sacrificed by decapitation. Blood samples were collected and distributed into EDTA centrifuge tubes. The serum was prepared by centrifugation (1500×g, 15 min, 4 °C), aliquotted into 1.5 ml vials and frozen at −80 °C for biochemical analyses. Liver, kidney, heart, pancreas and epididymal fat were carefully removed, weighed and then stored at −80 °C. Samples from liver (1 g) were homogenized in 2 ml of TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) and centrifuged at 9000 rpm for 15 min at 4 °C. The supernatants were collected and used for various biochemical estimations.

2.7. Determination of serum and liver biomarkers

2.7.1. Serum lipid levels

Concentrations of TC, TG and HDL-c in serum were determined by enzymatic colorimetric methods using available commercial kits (Biomaghreb, Tunisia). Serum TC was measured using a cholesterol oxidase assay, while serum TG was measured by the glycerol kinase assay. The resulting coloration intensity was measured at 505 nm and the results were expressed as mmol/l. The LDL-c and the very low-density lipoprotein cholesterol (VLDL-c) fractions were calculated according to the following equations:

$$\text{LDL-c} = \text{TC} - \text{HDL-c} - (\text{TG}/2.2) \text{ [22]}$$

$$\text{VLDL-c} = \text{TG}/2.2 \text{ [23]}.$$

To predict atherosclerosis and cardiovascular problems, atherogenic index (AI), atherogenic index of plasma (AIP) and coronary risk index (CRI) were determined referring to the equations given below:

$$\text{AI} = \text{LDL-c} / \text{HDL-c} \text{ [22]}$$

$$\text{AIP} = \log (\text{TG}/\text{HDL-c}) \text{ [24]}$$

$$\text{CRI} = \text{TC} / \text{HDL-c} \text{ [25]}.$$

2.7.2. Hepatic TC and TG levels

The TC and TG concentrations in the livers of all experimental rats were determined using the commercial kits from Biomaghreb, Tunisia. The results were expressed as µmol per g of liver tissue.

2.8. Pancreatic lipase activity

Pancreatic lipase activity was evaluated according to kinetic method using commercial kit (BIOLABO, France). The coloration intensity was measured at 550 nm and the residual enzymatic activity in each serum was calculated using the following equation:

$$\text{Lipase activity} = \frac{(\Delta \text{ abs/min) Calibrator} - (\Delta \text{ abs/min) Blank}}{(\Delta \text{ abs/min) Assay} - (\Delta \text{ abs/min) Blank}} \times \text{Calibrator}$$

where, (Δ abs/min)Assay: Rate of change per minute for the sample; (Δ abs/min)Blank: Rate of change per minute for the blank; (Δ abs/min)Calibrator: Rate of change per minute for Lipase Color Calibrator.

Lipase activity results were then expressed as U/l.

2.9. Measurement of pancreatic lipase activity *in vitro*

The method was modified from the assay reported by Nakai et al. [26], in which 4 methylumbelliferyl oleate (4-MU oleate) was used as a substrate to measure the pancreatic lipase inhibitory activity. Twenty-five microliters of a sample solution dissolved in water and 50 µL of a 0.1 mM 4-MU oleate solution dissolved in a buffer consisting of 13 mM Tris-HCl,

150 mM NaCl, and 1.3 mM $CaCl_2$ (pH 8.0) were mixed in the well of a microtiter plate, and 25 µl of the lipase solution (0.5 mg/ml) in the above buffer was then added to start the enzyme reaction. After incubation at 37 °C for 10 min, the amount of 4-methylumbelliferone released by lipase was measured with a fluorometrical microplate reader (Fluoroskan Ascent C Lab- Systems, Inc.) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The IC_{50} of the test sample was obtained from the least-squares regression line of the plots of the logarithm of the sample concentration (log) versus the pancreatic lipase activity (%).

2.10.   Histopathological examination

The pieces of liver, aorta and heart tissues from the different groups were fixed in a Bouin solution for 24 h, and then transported in a 10% formalin solution. The fixed tissues were embedded in paraffin and then sectioned at 4 µm thick. Sections were then stained with hematoxylin–eosin and examined under a Motic AE2000 light microscope.

2.11.   Statistical analysis

All data are presented as mean ± standard error of the mean (SEM) and analyzed using the Statistical SPSS ver. 17.0, professional edition. A one-way analysis of variance (ANOVA) was then performed and followed by Duncun's test to estimate the significance among the main effects at the 5% probability level. Significant differences (P<0.05) between means were identified by multiple comparisons across the six groups using least significant difference (LSD) procedures.

3. Results and discussion
3.1. Production and RP-HPLC profiles of FSPHs

In this study, two fermented sardinelle protein hydrolysates were prepared by *B. subtilis* 26 and *B. amyloliquefaciens* An6. The hydrolysis degree at the end of cultures are 21.56 and 24.30% for FSPH-A26 and FSPH-An6, respectively.

RP-HPLC efficiently separates peptides from protein hydrolysates, and it can give some indication about hydrophobic/hydrophilic peptide ratio [27]. Several researchers have employed this technique in studying tryptic hydrolysate from bovine β-lactoglobulin [28], crude protease extract hydrolysates from zebra blenny [29] or thornback ray (*Raja clavata*) muscle hydrolysates [30]. The RP-HPLC elution profiles of FSPHs are presented in Fig. 1.

Several peaks are detectable by RP-HPLC confirming the hydrolysis of the muscle protein and the generation of several peptides in FSPHs. Two aromatic amino acids, tyrosine and tryptophan, were run separately and their retention times (13.3 min and 26.03 min, respectively) were used to divide the area under the chromatograms into three zones. Zone 1 consisted of peptides eluted before tyrosine (hydrophilic peptides). Zone 2 comprised of peptides eluted between tyrosine and tryptophan (low hydrophobic peptides), while zone 3 comprised of those eluted after tryptophan (high hydrophobic peptides). The hydrolysates have a high content of hydrophobic peptides than hydrophilic and low hydrophobic ones. FSPH-An6 showed the highest content of hydrophobic peptides. The differences in RP-HPLC profiles of FSPHs could be essentially due to the difference in the specificity of enzymes produced during fermentation.

3.2. MALDI-ToF mass spectrometry analysis

MALDI-ToF mass spectrometry is a technique used in this study to determine the molecular masses of peptides generated during fermentative hydrolysis. Many studies reported the molecular weight distribution of protein hydrolysates using MALDI-ToF [31,32,33,30]. As shown in Fig. 2., and in comparison with undigested proteins, FSPHs were mainly constituted of low molecular weight peptides in the range of 150–900 Da.

3.3. Effects of FSPHs on body weight gain, organ weights and food intake of rats

No toxic effects were observed during the experimental period. The behavior of the animals was normal throughout the whole experiment. The growth parameters of the different group of rats, during the ten weeks, are shown in Table 2. Initial BW of rats were not significantly different among the different groups ($p > 0.05$). As reported in Table 2, the final BW and the weekly BW gain of rats fed a HCD were significantly higher than those of the control group ($p < 0.05$). In fact, the BW gain of the HCD group increased by 67.97% compared to the CD group which indicated that fructose and sheep fat diet induced a significant obesity. However, the growth parameters between the SMF, and FSPHs groups and the control group were not significant, indicating that the administration of sardinelle protein and its hydrolysates had no toxic effect at the experimental dose. Nevertheless, fluvastatine administration manifested remarkably lower BW gain compared to the control group.

Regarding the FI, there were no significant difference between the control and HCD group (p>0.05) (Table 2). The administration of Fluv, SMF, FSPH-A26 and FSPH-An6 to rats fed HCD reduced slightly the FI by 7%, 8% and 13%, respectively, compared to that of the HCD group (p<0.05). These results could be attributed to the fact that the proteins of SMF and its hydrolysates could act in the regulation of components of the FI control such as satiety and appetite suppressing effect or in the reduction of energy intake [34]. Calculated food efficiency ratio (FER) represents BW gain based on the amount of total FI. FERs were significantly lower in animals fed HCD and treated with Fluv or FSPHs, compared to that of the untreated HCD group.

As reported in Table 3, the relative organ weights (the ratio of organ to final body weight) of heart, pancreas, kidney and liver of all groups were not affected by the high-fat and fructose diet (p>0.05). Further, the relative organ weights of all experimental groups were similar. However, an increase in relative epididymal fat tissue size in HCD group by approximately 67% was observed compared to the control group (p<0.05). The oral administration of the Fluv, SMF, FSPH-A26 and FSPH-An6 could suppress fat accumulation, and reduce epididymal fats by 34%, 26%, 31% and 24%, respectively, in comparison with HCD rats.

3.4. Effect of FSPHs on serum lipid parameters

The *in vivo* hypolipidemic effects of SMF and its hydrolysates were studied. Values of lipid parameters at the end of the experiment are presented in Table 4. There was a significant increase in the serum TG, TC, LDL-c and VLDL-c levels in the HCD group by approximately 78%, 72%, 250% and 78%, respectively, compared to the control group (p<0.05). The obtained results indicates that HCD caused hyperlipidemia. Nevertheless, HCD did not influence HDL-c level. The elevations in serum of total TC, TG and LDL-c levels observed in HCD group are in agreement with those reported by Jeon et al. [35], Tauseef et al. [36] and Ktari et al [10]. The high levels of LDL-c found in HCD rats may be attributed to a down-regulation in LDL-c receptors by fat included in the diet [37].

Interestingly, oral administration of fluvastatin and FSPHs was found to attenuate lipid disorders as evidenced by decreased level of TC, TG, LDL-c and VLDL-c levels and the effects of FSPHs were more prominent than those of SMF.

Nevertheless, there were no significant differences in serum TC, TG and LDL-c levels between rats fed both protein hydrolysates. Indeed, compared to the HCD group, the serum

levels of TG, TC, LDL-c and VLDL-c in FSPH-A26 were decreased by 32%, 18%, 34% and 32%, respectively, and in FSPH-An6 by 40%, 23.5%, 43% and 40%, respectively.

Further, oral administration of Fluv and FSPHs increased the HDL-c levels compared to the corresponding values of HCD group and the value obtained were even higher than that of control group. Similar results were also reported by Lassoued et al. [11] with protein hydrolysates from *Boops boops* and Ktari et al. [10] with protein hydrolystes from *Salaria bascilisca*. Ben Khaled et al. [9] also reported that sardinelle protein hydrolysates obtained by enzymatic hydrolysis help to balance the serum lipid profile in rats fed a high-fat diet. A higher content of HDL-c is very important in humans because it is correlated with a reduced risk of coronary heart disease [38]. The increase of HDL-c facilitates the transport of cholesterol from the serum to the liver, where it is catabolized and excreted from the body.

However, SMF was found to reduce slightly TG, TC, LDL-c and VLDL-c values compared to those of rats fed HCD, and the values obtained were still higher than those of control group.

Atherogenic properties of the different treatments are also presented in Table 4. The atherogenic index (AI), defined as the ratio of LDL-c and HDL-c, is one of the most important useful predictors of atherosclerosis. A lower index value indicates a lower extent of atherosclerosis risk. In HCD group, AI increased by 3.6-fold respecting to control group. It was interesting to note that AI was significantly reduced in the Fluv and FSPHs groups compared to index value of HCD group, and the values were similar to those of control rats. However, SMF was less efficient than FSPHs.

Furthermore, the atherogenic index of plasma (AIP) and the coronory risk index (CRI) were conducted in this study. Treatment of obese rats with fluvastatin, SMF and FSPHs caused a significant reduction in AIP. Indeed, the AIP has decreased by 35% following treatment with Fluvastatin. The treatment with SMF the FSPH-A26 and FSPH-An6 reduced AIP by 25%, 49% and 60%, respectively. Similarly, the different types of treatments caused a significant reduction (22-45%) of the CRI compared to HCD rats.

The decrease in TG, TC and LDL-c and the increase in HDL-c could lower the risk of developing coronary heart disease. The results obtained indicate that the FSPHs, especially FSPH-An6 may have a very interesting cardioprotective potential.

3.5. Effect of FSPHs on liver TG and TC contents

TG and TC contents in liver tissue were also analyzed and the findings are shown in Table 5. As expected in comparison with rats fed a standard diet, those fed HCD showed a significant increase in TG (58%) and TC (149%) levels, whereas, the administration of Fluv, SMF and FSPHs reduced the lipids levels, which shows their hypolipidemic effects. Indeed, administration of SMF to rats fed HCD significantly reduced TG and TC values in liver tissue by 22% and 40%, respectively. FSPHs were more efficient than SMF in reduction of liver TG and TC concentrations, which were similar to those of Fluv group. Hepatic TG and TC concentrations were reduced by 34%, 34%, 38% and 53%, 46%, 44%, respectively, in rats fed HCD and administered with Fluv, FSPH-A26 and FSPH-An6, respectively, compared to that of HCD group.

So, all of these results suggest that FSPHs possesses a potential lipid-lowering effect in serum as well as in liver tissue, even after a long term of high-fat and fructose intake. The present result was similar to that of a previous study regarding the lowering lipid levels of salamon protein hydrolysates in rats fed high-cholesterol diet [15]. In another study, Hosomi et al. [39] reported that protein hydrolysates of Alaska pollock (*Theragra chalcogramma*) muscle induced the regulation of serum and hepatic TC levels.

These results indicated that FSPHs might have strong cardioprotective potential similar to that of Fluv drug.

3.6. Effects of FSPHs on pancreatic lipase activity *in vitro* and *in vivo*

The pancreatic lipase inhibitors represent one of the most prescribed drugs for the treatment of obesity. In fact the pancreatic lipase is a hydrolytic enzyme of dietary fats [40] and it is considered as the first responsible of gastrointestinal triglycerides absorption [41].

The effects of FSPHs on pancreatic lipase activity was first investigated *in vitro*. As shown in Table 6, both hydrolysates showed a significant and dose dependant inhibitory effect of lipase. The IC_{50} against the pancreatic lipase values of FSPH-A26 and FSPH-An6 were 1.17 mg/ml and 1.49 mg/ml, respectively, which are lower than that of sardinelle meat flour (3 mg/ml) and higher than that of fluvastatin (IC_{50} = 0.44 mg/ml). Zhang et al. [42] reported a higher pancreatic lipase inhibition rate of *T. officinale* extract with an IC_{50} of 78.2 µg/ml. In another study, Nakai et al. [26] described that the oolong tea polymerized polyphenols (OTPP) showed a stronger inhibition activity against pancreatic lipase (IC_{50}= 0.28 µg/ml).

The lipase activities in all experimental rat groups were also determined, and the results are shown in Fig. 3. After ten weeks of treatment, the lipase activity in the HCD group increased significantly by 47.6% compared to that of the control group ($p<0.05$). Oral administration of Fluv to HCD fed rats decreased the lipase activity by 15% compared to the HCD group; however, the value obtained was still higher than that of the control group. Interestingly, the administration of SMF and its hydrolysates resulted in a significant decrease in the lipase activity. The FSPH-A26 group showed markedly lower lipase activity than did the other groups and was 44% lower than that of HCD group. Further, the lipase activities of the FSPHs groups were obviously lower than that of the control group.

The obtained results are in agreement with several works reported the hypolipidemic effects of fish protein hydrolysates [43,11]. The results obtained indicate that sardinelle protein hydrolysates may induce hypolipidemic effect and lipid absorption and could prevent risk of coronary artery disease [44].

3.7. Histological examination

The findings obtained via biochemical assays were further confirmed by histopathological study. As shown in Fig. 4Ab, in HCD group, lipids accumulated in the hepatocytes as observable vacuoles. These vacuoles have a clear appearance as indicated by the arrows. The liver architectures of the rats treated with fluvastatin and the protein hydrolysates were noted to undergo marked improvements, indicating the ameliorative effect of peptides in FSPHs against hypercholesterol induced hepatic lipid accumulation. The animals treated with Fluv (Fig. 4Ac), FSPH-A26 (Fig. 4Ae) and FSPH-An6 (Fig. 4Af) showed that most of the hepatocytes were apparently normal, without lipid accumulations. SMF treatment did not produce a significant change in the histopathology of the liver tissue (Fig. 4Ad). These observations indicated that FSPHs could effectively inhibit liver fatty infiltration and reduce the formation of lipid droplets. Further, they confirmed our biochemical data showing a significant reduction of lipids in liver (Table 4).

Histological study of the tissue infarction of heart in HCD group revealed remarkable hypertrophy of cells, as indicated by the arrows (Fig 4Bb). In contrast, a significant reduction of this enlargement was observed in rats treated with SMF and FSPHs (Fig 4B d, e, f). This effect is similar to that of Fluv (Fig 4Bc) and corrects the perturbations induced by the high-fat and fructose diet.

Representative aortic transverse sections obtained from each group are shown in Fig. 4C. As shown in Fig. 4Ca, the aortic walls in the control rats were smooth and intact. This ordered organization was also observed in rats treated with Fluv, SMF and FSPHs (Fig. 4Cc, d, e and f, respectively). However, by feeding HCD, a thick layer of lipid deposition within intima of aorta, which is typical for atherosclerosis, was developed as shown in Fig. 4Cb. The HCD was also responsible for the development of lesion in the aortic wall.

4. Conclusion

The results show that FSPHs could suppress body weight gain and reduce the serum levels of TC, TG and LDL-c, and increase the level of HDL-c in rats fed high fat and fructose diet. Furthermore, FSPHs may have a strong cardioprotective effect demonstrated by a significant reduction of atherogenic indexes.

The results provide an important basis for developing the fermented sardinelle muscle protein hydrolysates as a natural additive in functional foods in order to protect against several obesity complications.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figure captions

Fig. 1. RP-HPLC profiles of SMF and FSPHs. The column was equilibrated with solvent A (0.1% TFA in ultrapure water) and peptides were eluted with a linear increase in solvent B (0.085% TFA in acetonitrile:water (60:40 v:v)) from 0% to 100%. SMF represents undigested sardinelle proteins; FSPH-A26 and FSPH-An6 represent sardinelle protein hydrolysates produced by fermentation using *B. subtilis* A26 and *B. amyloliquefaciens* An6, respectively.

Fig. 2. MALDI-Tof spectra of SMF and FSPHs meseared from 150 to 2000 Da. SMF represents undigested sardinelle proteins; FSPH-A26 and FSPH-An6 represent sardinelle protein hydrolysates produced by fermentation using *B. subtilis* A26 and *B. amyloliquefaciens* An6, respectively.

Fig. 3. Levels of pancreatic lipase activity in control and experimental groups of rats. CD represents control group; HCD represents the group fed hypercaloric diet; Fluv represents Fluvastatin; SMF represents undigested sardinelle proteins; FSPH-A26 and FSPH-An6 represent sardinelle protein hydrolysates produced by fermentation using *B. subtilis* A26 and *B. amyloliquefaciens* An6, respectively. Data are expressed as mean ± SEM of three rats in each group. [a, b, c] indicate significant differences compared to the values of CD, HCD and HCD + Fluv groups, respectively, at $p < 0.05$.

Fig. 4. Histopathology of liver tissue (×200) (A), heart (×200) (B) and aorta (×200) (C) of control and experimental animals. CD represents control group; HCD represents the group fed hypercaloric diet; Fluv represents Fluvastatin; SMF represents undigested sardinelle proteins; FSPH-A26 and FSPH-An6 represent sardinelle protein hydrolysates produced by fermentation using *B. subtilis* A26 and *B. amyloliquefaciens* An6, respectively.
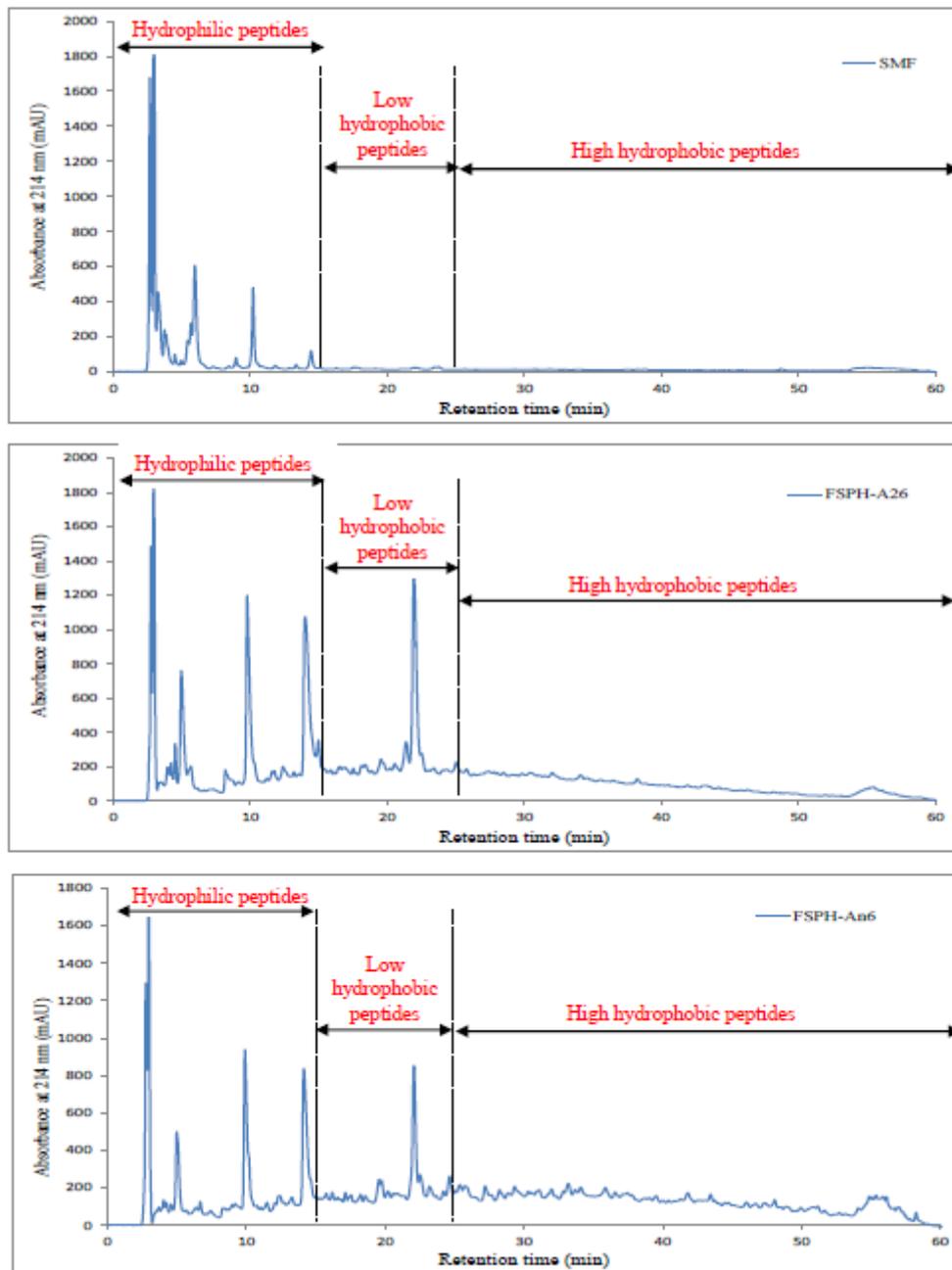
Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

A

(a) CD

(b) HCD

(c) HCD + Fluv

(d) HCD + SMF

(e) HCD + FSPH-A26

(f) HCD + FSPH-An6

B

(a) CD

(b) HCD

(c) HCD + Fluv

(d) HCD + SMF

(e) HCD + FSPH-A26

(f) HCD + FSPH-An6

C

(a) CD

(b) HCD

(c) HCD + Fluv

(d) HCD + SMF

(e) HCD + FSPH-A26

(f) HCD + FSPH-An6
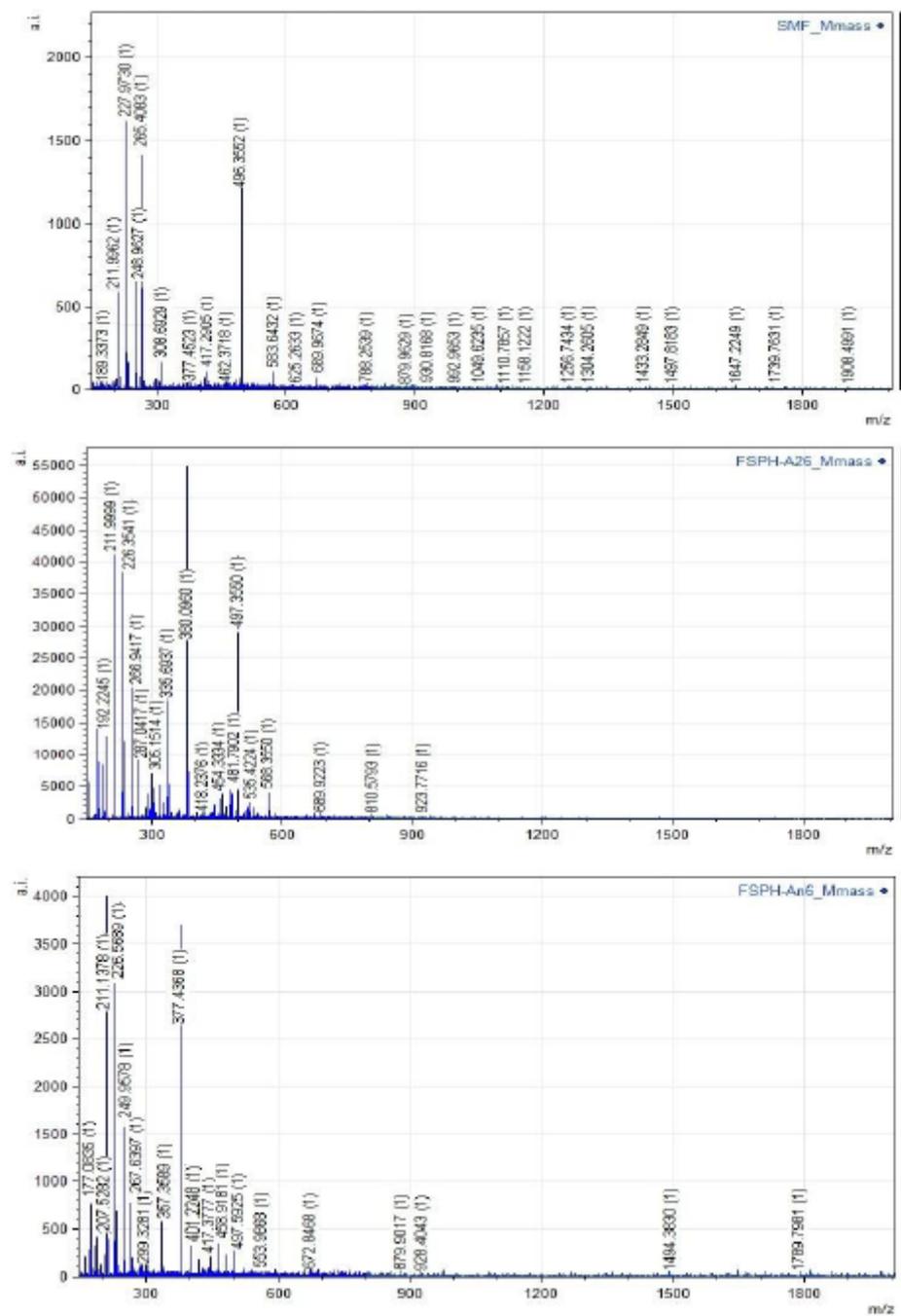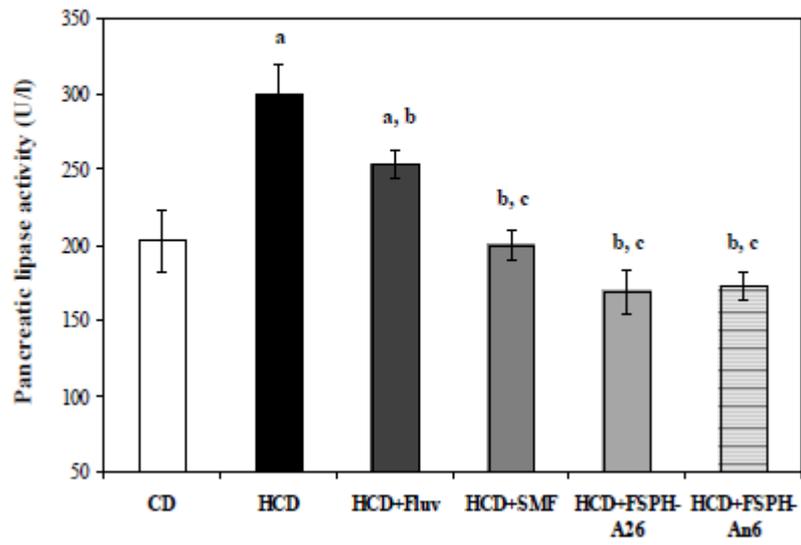
← → Alteration of elastin fibers

Table 1

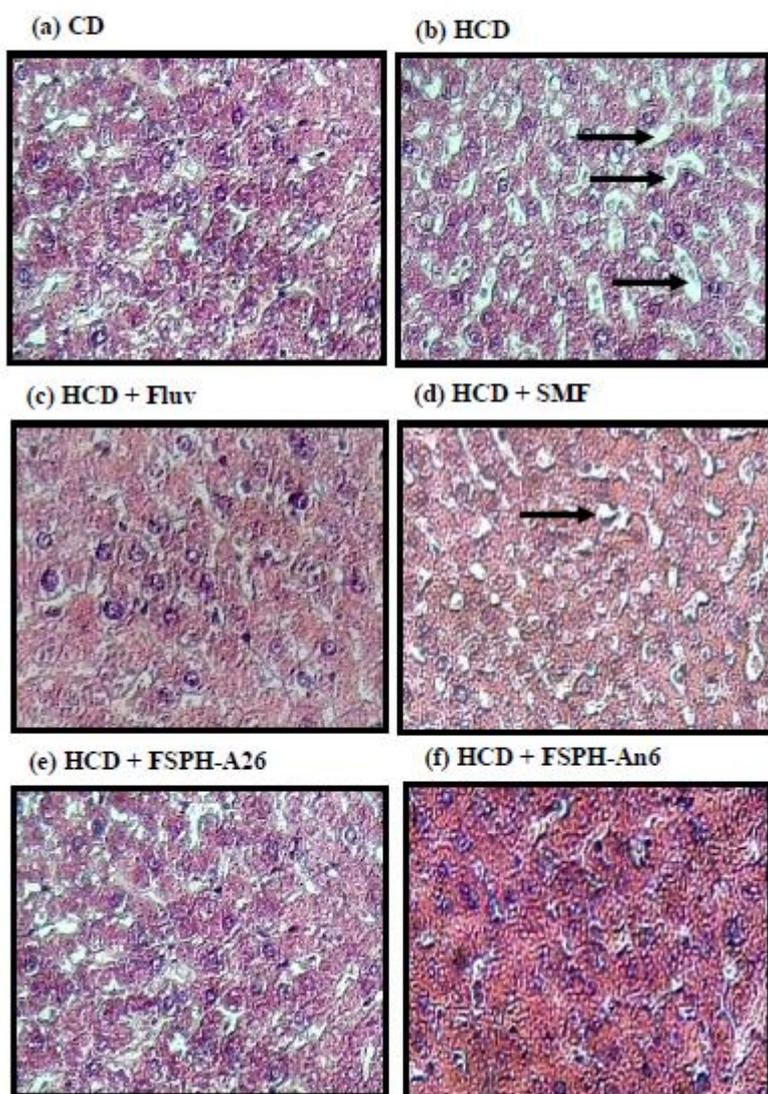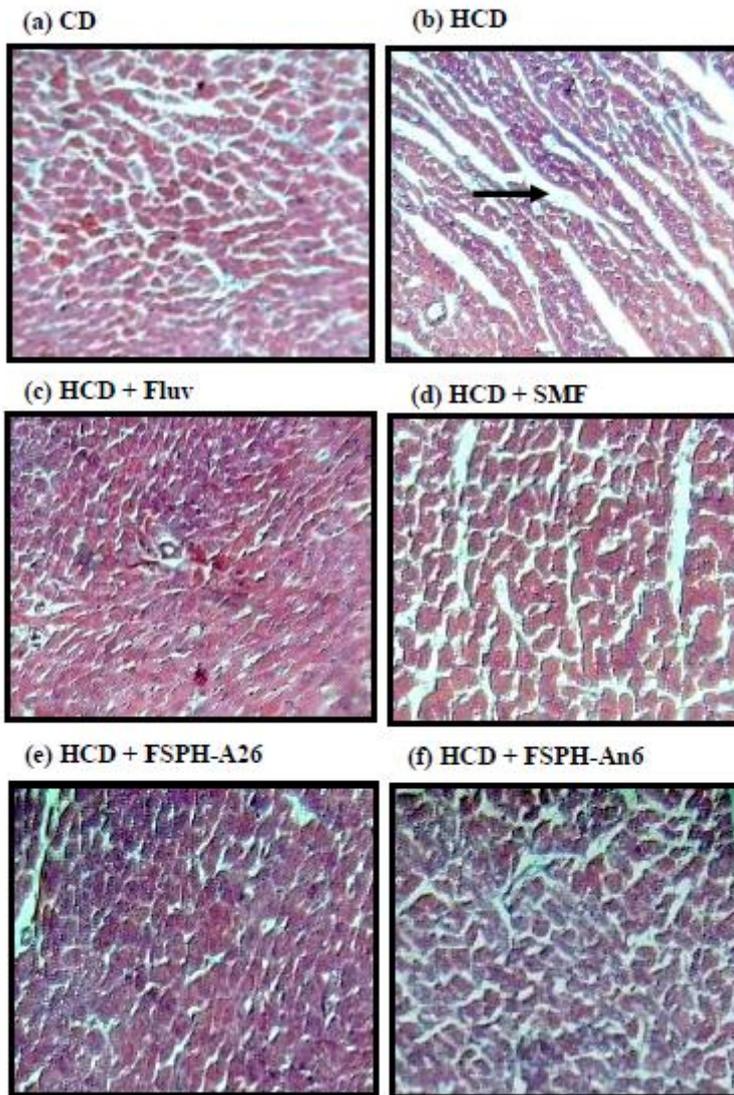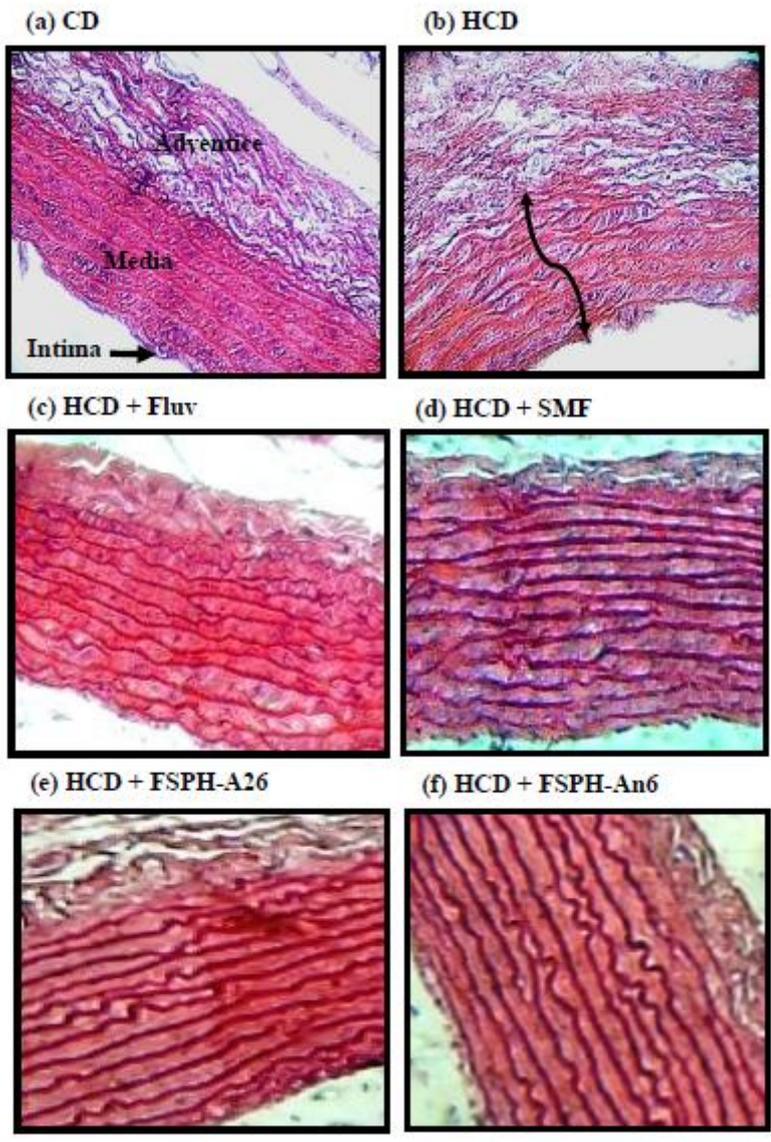Composition of the standard diet. This food consists of corn, soya, VMC (vitamins minerals compound) with the following characteristics.

Nutritional properties (%)	
Moisture (maximal)	14
Fibers (maximal)	3.4
Proteins (minimal)	22
Fat (maximal)	3.5
Ash (maximal)	6.7
Carbohydrate	46.5
Calorific value (kcal/kg)	2850
Amino acid (%)	
Methionine	0.60
Cysteine	0.38
Threonine	0.80
Tryptophan	0.30
Mineral mix (mg/kg)	
Manganese	80
Fer	50
Cuivre	18.75
Zinc	65
Selenium	0.3
Cobalt	0.2
Iode	1.2
Vitamin and antioxidant (mg/kg)	
Vitamine A	13.000
Vitamine D3	4375
Vitamine H	62.5
Antioxidant (BHA–BHT)	125

Table 2

Effects of different treatments on body weight gain and food intake.

	CD	HCD	HCD + Fluv	HCD + SMF	HCD + FSPH-A26	HCD + FSPH-An6
Initial body weight (g/rat)	150	146	156	148	145	151
Final body weight (g/rat)	224.4 ±8.44	275.75 ±11.24	222.00 ±21.89 [b]	235.5 ±15.45	230.20 ±10.24	238.60 ±10.70
Body weight gain (g/rat/week)	7.44± 0.84	12.97 ± 1.12[a]	6.60 ± 2.20 [a, b]	8.75 ± 1.54 [b]	8.52 ± 0.87 [b]	8.76 ±1.07 [b]
Food intake (g/rat/week)	95.00 ± 1.87	93.04± 2.34	87.95± 1.45 [a, b]	86.36±1.06[a, b]	85.79± 1.80 [a, b]	87.33±1.58 [a,b]
Food efficiency ratio (%)	7.83 ± 0.45	13.94 ± 0.48[a]	7.50 ± 1.52 [b]	10.13 ± 1.45	9.93 ± 0.48	10.03 ± 0.68

CD represents control group; HCD represents the group fed hypercaloric diet; Fluv represents Fluvastatin; SMF represents undigested sardinelle proteins; FSPH-A26 and FSPH-An6 represent sardinelle protein hydrolysates produced by fermentation using *B. subtilis* A26 and *B. amyloliquefaciens* An6, respectively.

Food efficiency ratio (FER) = body weight gain (g) $\times$ 100 / food intake (g).

Values are expressed as mean ± SEM (mean of six determinations).

[a, b, c] indicate significant differences compared to the values of CD, HCD and HCD + Fluv groups, respectively, at p<0.05.

Table 3

Effects of different treatments on relative organ weights of rats.

Relative organ weights (g/100 g of body weight)	CD	HCD	HCD + Fluv	HCD + SMF	HCD + FSPH-A26	HCD + FSPH-An6
Epididymal fat	0.64± 0.05	1.07± 0.35[a]	0.71± 0.09	0.79± 0.08	0.74± 0.13	0.81± 0.13
Heart	0.40±0.03	0.39±0.07	0.33±0.03	0.37±0.02	0.38±0.03	0.38±0.01
Pancreas	0.25± 0.04	0.32± 0.05	0.27± 0.01	0.25± 0.05	0.30± 0.02	0.26± 0.02
Kidney	0.77± 0.05	0.67± 0.02	0.67± 0.07	0.66± 0.05	0.66± 0.02	0.68± 0.01
Liver	3.14± 0.34	2.95± 0.22	2.72± 0.27	3.27± 0.12	2.96± 0.16	3.33± 0.28

CD represents control group; HCD represents the group fed hypercaloric diet; Fluv represents Fluvastatin; SMF represents undigested sardinelle proteins; FSPH-A26 and FSPH-An6 represent sardinelle protein hydrolysates produced by fermentation using *B. subtilis* A26 and *B. amyloliquefaciens* An6, respectively.

Values are expressed as mean ± SEM (mean of six determinations).

indicates significant differences compared to the values of CD, at $p<0.05$.

Table 4
Serum lipid profile in control and experimental groups of rats.

	CD	HCD	HCD + Fluv	HCD + SMF	HCD + FSPH-A26	HCD + FSPH-An6
TG (mmol/l)	1.27± 0.16	2.26 ± 0.13 [a]	1.80 ± 0.06 [a]	1.89 ± 0.23 [a]	1.54 ± 0.16 [b]	1.36 ± 0.09 [b]
TC (mmol/l)	1.34± 0.01	2.30 ± 0.09 [a]	1.80 ± 0.06 [a, b]	1.94 ± 0.07 [a, b]	1.88 ± 0.07 [a, b]	1.76 ± 0.09[a, b]
HDL-c (mmol/l)	0.55± 0.01	0.52 ± 0.02	0.69 ± 0.02 [a, b]	0.56 ± 0.01[c]	0.68 ± 0.04[b]	0.75 ± 0.09[a, b]
LDL-c (mmol/l)	0.22 ± 0.01	0.77 ± 0.05[a]	0.29 ± 0.07 [b]	0.52 ± 0.14 [a]	0.51 ± 0.06	0.44 ± 0.04
VLDL-c (mmol/l)	0.58 ± 0.07	1.03 ± 0.06[a]	0.82 ± 0.03[a]	0.86 ± 0.10 [a]	0.70 ± 0.07 [b]	0.62 ± 0.04 [b]
AI	0.40 ± 0.02	1.44 ± 0.08[a]	0.42 ± 0.10 [b]	0.92 ± 0.25[a, b, c]	0.77 ± 0.14 [b]	0.52 ± 0.11 [b]
AIP	0.39 ± 0.05	0.63 ± 0.01[a]	0.41± 0.03 [b]	0.47 ± 0.06	0.32 ± 0.08 [b]	0.25 ±0.07[b]
CRI	2.45 ± 0.01	4.43 ± 0.05[a]	2.60 ± 0.05[b]	3.46 ± 0.14 [a, b, c]	2.73 ± 0.26 [b]	2.43 ± 0.24[b]

CD represents control group; HCD represents the group fed hypercaloric diet; Fluv represents Fluvastatin; SMF represents undigested sardinelle proteins; FSPH-A26 and FSPH-An6 represent sardinelle protein hydrolysates produced by fermentation using *B. subtilis* A26 and *B. amyloliquefaciens* An6, respectively.

TG : triglycerides ; TC : total cholesterol ; HDL-c : high-density lipoprotein cholesterol ; LDL-c : low-density lipoprotein cholesterol ; VLDL-c: very low-density lipoprotein cholesterol ; AI : atherogenic index; AIP : atherogenic index of plasma; CRI : coronory artery risk index

LDL-c = CT − (HDL-c + TG/2,2) ; VLDL-c = TG/2.2 ; AI = LDL-c/HDL-c ; AIP = log (TG/HDL-c) ; CRI = TC/HDL-c.

Values are expressed as mean ± SEM (mean of six determinations).

[a, b, c] indicate significant differences compared to the values of CD, HCD and HCD + Fluv groups, respectively, at p<0.05.

Table 5

Hepatic lipid profile in control and experimental groups of rats.

	CD	HCD	HCD + Fluv	HCD + SMF	HCD + FSPH-A26	HCD + FSPH-An6
TG (µmol/g tissue)	4.41 ± 0.11	6.97 ± 0.35 [a]	4.57 ± 0.15 [b]	5.42 ± 0.69 [b]	4.57 ± 0.23 [b]	4.33 ± 0.23 [b]
TC (µmol/g tissue)	6.92 ± 0.42	17.21 ± 0.51 [a]	8.16 ± 0.51 [b]	10.26 ± 0.36 [a, b, c]	9.26 ± 0.61 [a, b]	9.73 ± 0.43 [a, b]

CD represents control group; HCD represents the group fed hypercaloric diet; Fluv represents Fluvastatin; SMF represents undigested sardinelle proteins; FSPH-A26 and FSPH-An6 represent sardinelle protein hydrolysates produced by fermentation using *B. subtilis* A26 and *B. amyloliquefaciens* An6, respectively.

Values are expressed as mean ± SEM (mean of six determinations).

[a, b, c] indicate significant differences compared to the values of CD, HCD and HCD + Fluv groups, respectively, at $p < 0.05$.

Table 6
In vitro pancreatic lipase inhibition rate IC_{50} of sardinelle meat flour and their hydrolysates (FSPH-A26 and FSPH-An6).

Inhibitor	Concentration (mg/ml)	Pancreatic lipase inhibition (%)	IC_{50} (mg/ml)
Fluvastatin	0.25	25.85 ± 1.34	
	0.50	62.95 ± 4.04	0.44 ± 0.01
	0.75	81.86 ± 0.43	
SMF	1	8.00 ± 0.71	
	2	23.43 ± 0.60	3.00 ± 0.07
	3	43.06 ± 3.96	
	4	73.32 ± 1.58	
FSPH-A26	0.5	31.29 ± 1.36	
	1	42.80 ± 1.81	1.17 ± 0.08
	2	76.32 ± 0.51	
	3	99.48 ± 0.37	
FSPH-An6	0.5	27.82 ± 0.24	
	1	36.86 ± 1.04	1.49 ± 0.03
	2	65.08 ± 1.43	
	3	81.31 ± 1.87	

SMF represents undigested sardinelle proteins; FSPH-A26 and FSPH-An6 represent sardinelle protein hydrolysates produced by fermentation using *B. subtilis* A26 and *B. amyloliquefaciens* An6, respectively.

Values are given as mean ± SD from duplicate determinations (n= 2).

Highlights

- Fermented sardinelle protein hydrolysates (FSPHs) were prepared by two proteolytic bacteria;

- Hypercaloric diet (HCD) induced obesity and hyperlipidemia;

- FSPHs were found to attenuate obesity and hyperlipidemia in HCD-fed rats;

- FSPHs exhibited a potent cardioprotective effect;

- FSPHs could be used as ingredients to formulate functional foods.