

A process of forming meat-based seasoningTechnical Field

The present invention relates to a process of forming meat-based seasoning.

5 Background

The by-products at animal slaughterhouses or meat manufacturing industries is often discarded as waste food or reused as low value products such as animal feeds and fertilizers. Most of these meat-based by-products contain a significant amount of nutrients, including lipids, proteins, peptides, free amino acids, vitamins and minerals.

10 There is therefore a need to reduce food waste and convert them into useful products.

Summary of the invention

The present invention seeks to address these problems, and/or to provide an improved process of forming meat-based seasoning.

15 According to a first aspect, the present invention provides a process of forming meat-based seasoning, comprising fermenting meat-based hydrolysate with a non-halophilic microorganism to form the meat-based seasoning. According to a particular aspect, the process does not comprise addition of salt.

The non-halophilic microorganism may be any suitable microorganism. For example, the non-halophilic microorganism may be, but not limited to, non-halophilic yeast, non-halophilic lactic acid bacteria, or a combination thereof.

20 According to a particular aspect, the non-halophilic microorganism may be non-halophilic yeast.

According to a particular aspect, the non-halophilic microorganism may be non-halophilic lactic acid bacteria. In particular, the non-halophilic lactic acid bacteria may be, but not limited to: a probiotic lactic acid bacteria, a dairy lactic acid bacteria, or a combination thereof.

25 The fermenting may comprise fermenting meat-based hydrolysate for a suitable period of time. For example, the fermenting may comprise fermenting meat-based hydrolysates for 1-20 days.

The fermenting may comprise fermenting meat-based hydrolysate at a suitable temperature. For example, the fermenting may comprise fermenting at a temperature of 15-45°C.

According to a particular aspect, the fermenting may comprise fermenting meat-based hydrolysate with a non-halophilic yeast for 1-20 days. The fermenting may be at a temperature of 15-40°C.

According to another particular aspect, the fermenting may comprise fermenting meat-based hydrolysate with a non-halophilic lactic acid bacteria for 1-7 days. The fermenting may be at a temperature of 15-45°C.

According to another particular aspect, the fermenting may comprise fermenting meat-based hydrolysate with non-halophilic yeast and non-halophilic lactic acid bacteria. For example, the fermenting may comprise sequentially fermenting meat-based hydrolysate with non-halophilic yeast and non-halophilic lactic acid bacteria, or the fermenting may comprise fermenting meat-based hydrolysate with a co-inoculation of non-halophilic yeast and non-halophilic lactic acid bacteria.

The fermenting may further comprises adding fermentable sugar.

According to another particular aspect, the process may further comprise hydrolysing a meat-based mixture in the presence of an enzyme to form meat-based hydrolysate prior to the fermenting. The enzyme may be any suitable hydrolysing enzyme. For example, the enzyme may be, but not limited to, a protease.

The hydrolysing may be under suitable conditions. For example, the hydrolysing may be at a suitable pH and temperature. In particular, the hydrolysing may be at a pH of 5.5-7.5. In particular, the hydrolysing may be at a temperature of 45-60°C.

The process may further comprise heat treating the meat-based seasoning following the fermenting. The heat-treating may be under suitable conditions. For example, the heat-treating may be at a suitable temperature and pH and for a suitable period of time. In particular, the heat treating may be at a temperature of 90-121°C. In particular, the heat treating may be for 30-120 minutes. In particular, the heat treating may be at a pH of 4-6.

According to a second aspect, the present invention provides a meat-based seasoning prepared from the process of the first aspect.

According to a third aspect, there is also provided a meat-based seasoning comprising 10-45 mg/mL free amino acid. In particular, the meat-based seasoning may comprise a sodium content of ≤ 2 wt % based on total weight of the meat-based seasoning.

Brief Description of the Drawings

In order that the invention may be fully understood and readily put into practical effect there shall now be described by way of non-limitative example only exemplary embodiments, the description being with reference to the accompanying illustrative drawings. In the drawings:

Figure 1 shows a schematic representation of a process according to one embodiment of the present invention;

Figure 2 shows a schematic representation of a part of a process according to one embodiment of the present invention;

Figure 3 shows a schematic representation of a part of a process according to one embodiment of the present invention;

Figure 4 shows a schematic representation of a part of a process according to one embodiment of the present invention;

Figure 5 (a) shows degree of hydrolysis (DH) during enzymatic hydrolysis; Figure 5 (b) shows protein recovery after 6 hours of hydrolysis using four proteases (n=3);

Figure 6 (a) shows yeast cell counts changes over time; Figure 6 (b) shows pH changes of pork trimmings hydrolysate over time, of pork trimmings hydrolysate fermented by *Candida versatilis* NCYC1433, *Torulaspora delbrueckii* Prelude, *Saccharomyces cerevisiae* MERIT.ferm, and *Pichia kluyveri* FrootZen. ^{a,b,c}: Values within the same sample on different days followed by the same letters are not significantly different ($P > 0.05$). ^{A, B, C}: Values between samples on the same fermentation days followed by the same letters are not significantly different ($P > 0.05$);

Figure 7 shows changes in total reducing sugar and organic acids in pork trimmings hydrolysate fermented by *Candida versatilis* NCYC1433, *Torulaspora delbrueckii* Prelude, *Saccharomyces cerevisiae* MERIT.ferm, and *Pichia kluyveri* FrootZen. ^{a,b,c}: Values within the same sample on different days followed by the same letters are not significantly different ($P > 0.05$). ^{A, B, C}: Values between samples on the same fermentation days followed by the same letters are not significantly different ($P > 0.05$);

Figure 8 shows changes in GC-FID peak areas of (a) acids; (b) ethanol; (c) alcohols excluding ethanol; (d) aldehydes; (e) ketones; (f) esters; (g) furans, in pork trimmings hydrolysate fermented by yeasts 1433, Prelude, Merit, and FrootZen. ^{a,b,c}: Values within the same sample on different days followed by the same letters are not significantly different ($P > 0.05$). ^{A, B, C}: Values between samples on the same fermentation days followed by the same letters are not significantly different ($P > 0.05$);

Figure 9 (a) shows lactic acid bacterial (LAB) cell counts changes over time; Figure 9 (b) shows pH changes of pork trimmings hydrolysate over time, of pork trimmings hydrolysates fermented by *Lactobacillus fermentum* PCC, *Lb. plantarum* 299V, *Lactococcus lactis* subsp. *cremoris* DSM20069, *Lb. rhamnosus* GG, *Lb. acidophilus* NCFM, and *Lb. casei* Shirota. ^{a,b,c}: Values within the same sample on different days followed by the same letters are not significantly different ($P > 0.05$). ^{A, B, C}: Values between samples on the same fermentation days followed by the same letters are not significantly different ($P > 0.05$);

Figure 10 shows changes in total reducing sugar and organic acids in pork trimmings hydrolysate fermented by lactic acid bacteria *Lactobacillus fermentum* PCC, *Lb. plantarum* 299V, *Lactococcus lactis* subsp. *cremoris* DSM20069, *Lb. rhamnosus* GG, *Lb. acidophilus* NCFM, and *Lb. casei* Shirota. ^{a,b,c}: Values within the same sample on different days followed by the same letters are not significantly different ($P > 0.05$). ^{A, B, C}: Values between samples on the same fermentation days followed by the same letters are not significantly different ($P > 0.05$);

Figure 11 shows changes in GC-FID peak areas of (a) acids; (b) alcohols; (c) aldehydes; (d) ketones; (e) furans, in meat sauce samples fermented by lactic acid bacteria *Lactobacillus fermentum* PCC, *Lb. plantarum* 299V, *Lactococcus lactis* subsp. *cremoris* DSM20069, *Lb. rhamnosus* GG, *Lb. acidophilus* NCFM, and *Lb. casei* Shirota. ^{a,b,c}: Values within the same sample on different days followed by the same

letters are not significantly different ($P > 0.05$). ^{A, B, C}: Values between samples on the same fermentation days followed by the same letters are not significantly different ($P > 0.05$);

Figure 12 shows changes in pH and growth of *Lactobacillus fermentum* and *Pichia kluyveri* during pork hydrolysate fermentation for (a) single culture of *L. fermentum*, (b) single culture of *P. kluyveri*, (c) co-inoculation, and (d) sequential inoculation;

Figure 13 shows changes in amount of (a) glucose; (b) succinic acid; (c) lactic acid; (d) acetic acid during pork hydrolysate fermentation with different inoculation sequences of *L. fermentum* and *P. kluyveri*;

Figure 14 shows changes in GC-FID peak areas of (a) ethanol; (b) acetic acid; (c) hexanal; (d) 1-hexanol; (e) isoamyl acetate; (f) ethyl acetate; (g) hexyl acetate; (h) 2-phenylethyl acetate; and (i) phenylethyl alcohol;

Figure 15 shows changes of pH in control (0 °C) and pork hydrolysates with adjusted pH to (a) 5.5; and (b) 4.5 during the heat treatment at 90, 95 and 100 °C. All values are mean $\pm$ standard deviation with three replications of each sample treatment ($n = 3$);

Figure 16 shows amount of sugar reduction (mg/mL) in pork hydrolysates with adjusted pH to (a) 5.5; and (b) 4.5 during the heat treatment at 90, 95 and 100 °C. All values are mean $\pm$ standard deviation with three replications of each sample treatment ($n = 3$);

Figure 17 shows changes in amount of (a) proline in control and in each heat-treated sample at pH 5.5; (b) proline in control and in each heat-treated sample at pH 4.5; (c) cysteine in control and in each heat-treated sample at pH 5.5; (d) cysteine in control and in each heat-treated sample at pH 4.5; (e) methionine in control and in each heat-treated sample at pH 5.5; (f) methionine in control and in each heat-treated sample at pH 4.5; (g) phenylalanine in control and in each heat-treated sample at pH 5.5; (h) phenylalanine in control and in each heat-treated sample at pH 4.5; (i) total free amino acid contents in control and in each heat-treated sample at pH 5.5; (j) total free amino acid contents in control and in each heat-treated sample at pH 4.5. All values are mean $\pm$ standard deviation with three replications of each sample treatment ($n = 3$); and

Figure 18 shows changes of GC-MS/FID peak areas of (a) hexanal in control and in each heat-treated sample at pH 5.5; (b) hexanal in control and in each heat-treated

sample at pH 4.5; (c) furfural in control and in each heat-treated sample at pH 5.5; (d) furfural in control and in each heat-treated sample at pH 4.5; (e) 2-pentylfuran in control and in each heat-treated sample at pH 5.5; (f) 2-pentylfuran in control and in each heat-treated sample at pH 4.5. All values are mean $\pm$ standard deviation with three
5 replications of each sample treatment (n = 3).

Detailed Description

As explained above, there is a need for a way of reducing food wastage, and using the meat-based by-products in a more useful manner. The present invention provides a process for utilising such meat-based by-products and converting them into a meat-based seasoning.
10

In general terms, the present invention provides a process of forming a meat-based seasoning with high nutrient content and acceptable flavour profiles from meat-based by-products. In particular, the process of the present invention provides use of meat-based by-products and converting them into a meat-based seasoning such that the
15 process does not comprise addition of salt, while maintaining a suitable flavour profile and is able to achieve the conversion within a shorter period of time.

According to a first aspect, the present invention provides a process of forming meat-based seasoning, comprising fermenting meat-based hydrolysate with a non-halophilic microorganism to form the meat-based seasoning.

20 According to a particular aspect, the process does not comprise addition of salt. In particular, the process does not comprise the addition of any sodium chloride-containing salts. In this way, the meat-based seasoning formed from the process is much healthier since it comprises reduced or zero levels of added salts and thus, risk of hypertension and cardiovascular diseases of consumers of the meat-based
25 seasoning is reduced.

For the purposes of the present invention, meat-based refers to non-seafood meat-based products. According to a particular aspect, the meat-based hydrolysate may comprise hydrolysate of a meat-based mixture derived from non-seafood meat and/or non-seafood by-products such as, but not limited to, blood, bones, skin, meat trimming,
30 and/or fatty tissues of non-seafood meat. The non-seafood meat may be, but not

limited to, pork, mutton, beef, poultry, or a combination thereof. According to a particular aspect, the meat-based hydrolysate may be in liquid form.

The non-halophilic microorganism may be any suitable microorganism. For the purposes of the present invention, a non-halophilic microorganism is defined as a
5 microorganism that is able to grow in low or no salt containing media, for example in media comprising salt concentration of ≤ 0.2 mol/L. The non-halophilic microorganism may be, but not limited to, non-halophilic yeast, non-halophilic lactic acid bacteria, or a combination thereof.

According to a particular aspect, the non-halophilic microorganism may be non-
10 halophilic yeast. For example, the non-halophilic yeast may be, but not limited to: *Torulaspora delbrueckii* (*T. delbrueckii*), *Saccharomyces cerevisiae* (*S. cerevisiae*), *Pichia kluyveri* (*P. kluyveri*), or a combination thereof. In particular, the non-halophilic yeast may be, but not limited to, *T. delbrueckii* Prelude, *S. cerevisiae* MERIT, *P. kluyveri* FrootZen, or a combination thereof. Even more in particular, the non-halophilic
15 yeast may be *P. kluyveri*.

According to a particular aspect, the non-halophilic microorganism may be non-halophilic lactic acid bacteria. In particular, the non-halophilic lactic acid bacteria may be, but not limited to: a probiotic lactic acid bacteria, a dairy lactic acid bacteria, or a combination thereof. For example, the non-halophilic lactic acid bacteria may be, but
20 not limited to: *Lactobacillus fermentum* (*Lb. fermentum*), *Lactobacillus rhamnosus* (*Lb. rhamnosus*), *Lactobacillus plantarum* (*Lb. plantarum*), *Lactobacillus acidophilus* (*Lb. acidophilus*), *Lactobacillus casei* (*Lb. casei*), *Lactococcus lactis* subsp. *cremoris*, or a combination thereof. Even more in particular, the non-halophilic lactic acid bacteria is *Lb. fermentum*.

25 According to another particular aspect, the fermenting may comprise fermenting meat-based hydrolysate with non-halophilic yeast and non-halophilic lactic acid bacteria. For example, the fermenting may comprise sequentially fermenting meat-based hydrolysate with non-halophilic yeast and non-halophilic lactic acid bacteria. In particular, the fermenting may comprise fermenting the meat-based hydrolysate with
30 non-halophilic yeast followed by fermenting the meat-based hydrolysate with non-halophilic lactic acid bacteria. Alternatively, the fermenting may comprise fermenting the meat-based hydrolysate with non-halophilic lactic acid bacteria followed by

fermenting the meat-based hydrolysate with non-halophilic yeast. In particular, the fermenting may comprise fermenting the meat-based hydrolysate with non-halophilic lactic acid bacteria followed by fermenting the meat-based hydrolysate with non-halophilic yeast, wherein the non-halophilic lactic acid bacteria may be *Lb. fermentum* and the non-halophilic yeast may be *P. kluyveri*.

According to another particular aspect, the fermenting may comprise fermenting meat-based hydrolysate with a co-inoculation of non-halophilic yeast and non-halophilic lactic acid bacteria. The non-halophilic yeast and the non-halophilic lactic acid bacteria may be as described above. In particular, the non-halophilic lactic acid bacteria may be *Lb. fermentum* and the non-halophilic yeast may be *P. kluyveri*.

The fermenting may comprise fermenting the meat-based hydrolysate with a suitable amount of the non-halophilic microorganism. According to a particular aspect, the fermenting may comprise adding non-halophilic microorganism to obtain an increased microorganism live count during fermentation of at least 1 log CFU/mL. For example, the amount of non-halophilic microorganism added may be at least 4 log CFU/mL. In particular, the amount of non-halophilic microorganism added may be about 5-8 log CFU/mL, 6-7 log CFU/mL. Even more in particular, the amount of non-halophilic yeast added may be 5-6 log CFU/mL. Even more in particular, the amount of non-halophilic lactic acid bacteria added may be 6-7 log CFU/mL.

The fermenting may comprise fermenting the meat-based hydrolysates under suitable conditions. The fermenting may comprise fermenting meat-based hydrolysate for a suitable period of time. For example, the fermenting may comprise fermenting meat-based hydrolysates for 1-20 days. In particular, the fermenting may comprise fermenting for 2-18 days, 3-15 days, 5-12 days, 7-10 days, 8-9 days.

According to a particular aspect, when the non-halophilic microorganism is a non-halophilic yeast, the fermenting may be for 1-20 days. In particular, the fermenting may be for 2-18 days, 3-15 days, 4-14 days, 5-12 days, 6-11 days, 7-10 days, 8-9 days.

According to a particular aspect, when the non-halophilic microorganism is a non-halophilic lactic acid bacteria, the fermenting may be for 1-7 days. In particular, the fermenting may be for 1-6 days, 2-5 days, 3-4 days. Even more in particular, the fermenting may be for 1-5 days.

According to a particular aspect, when the non-halophilic microorganism is a non-halophilic yeast and a non-halophilic lactic acid bacteria, the fermenting may be for 1-25 days. In particular, when the fermenting comprises sequentially fermenting meat-based hydrolysate with non-halophilic yeast and non-halophilic lactic acid bacteria, the fermenting may be for 2-25 days. For example, the fermenting may be for 3-22 days, 5-20 days, 6-18 days, 7-15 days, 8-12 days. Even more in particular, the fermenting may be for 6-7 days.

When the fermenting meat-based hydrolysate comprises co-inoculation of non-halophilic yeast and non-halophilic lactic acid bacteria, the fermenting may be for 1-20 days. For example, the fermenting may be for 2-18 days, 3-15 days, 4-14 days, 5-12 days, 6-11 days, 7-10 days, 8-9 days. Even more in particular, the fermenting may be for 5-6 days.

The fermenting may comprise fermenting meat-based hydrolysate at a suitable period temperature. For example, the fermenting may comprise fermenting at a temperature of 15-45°C.

According to a particular aspect, when the non-halophilic microorganism is a non-halophilic yeast, the fermenting may be at a temperature of 15-40°C. For example, the fermenting may be at a temperature of 17-38°C, 18-35°C, 20-32°C, 22-30°C, 25-28°C. Even more in particular, the temperature may be 20-35°C.

According to another particular aspect, when the non-halophilic microorganism is a non-halophilic lactic acid bacteria, the fermenting may be at a temperature of 15-45°C. For example, the fermenting may be at a temperature of 16-40°C, 17-38°C, 18-35°C, 20-32°C, 22-30°C, 25-28°C. Even more in particular, the temperature may be 25-40°C.

The fermenting may further comprise adding fermentable sugar to the meat-based hydrolysate. The fermentable sugar may be any suitable fermentable sugar. For example, the fermentable sugar may be, but not limited to, glucose, fructose, lactose, or a combination thereof. Any suitable amount of fermentable sugar may be added. For example, the amount of fermentable sugar added may be 0.5-5 wt % based on the overall weight of the fermentable sugar and meat-based hydrolysate. In particular, the amount of fermentable sugar added may be 0.5-5 wt %, 1-4 wt %, 2-3 wt %. Even

more in particular, the amount may be 1-2 wt % based on the overall weight of the glucose and meat-based hydrolysate.

According to a particular aspect, the method may further comprise heat-treating the meat-based hydrolysate prior to adding the non-halophilic microorganism. For example, the heat-treating may comprise mild pasteurization or sterilisation of the meat-based hydrolysate. The heat-treating may extend the shelf life of the meat-based seasoning and may also reduce the risk of contamination during the method of forming the meat-based hydrolysate. In particular, the heat-treating may remove undesirable microorganisms prior to the adding of non-halophilic microorganism.

The heat-treating may be carried out under suitable conditions. For example, the heat-treating may be carried out at a temperature of about 60-90°C. In particular, the temperature may be about 80-90°C. Even more in particular, the temperature may be about 85°C.

The heat-treating may be carried out for a suitable period of time. The time for which heat-treating is carried out may depend on the temperature at which heat-treating is carried out. For example, the heat-treating may be for –10-45 minutes. In particular, the heat-treating may be for about –15-20 minutes. Even more in particular, the heat-treating may be for about 15 minutes.

The method may further comprise cooling the meat-based hydrolysate prior to adding the non-halophilic microorganism, and particularly if the meat-based hydrolysate underwent heat-treating as described above. In particular, the cooling may comprise cooling the meat-based hydrolysate to ambient temperature, for example about 25°C.

According to another particular aspect, the process may further comprise hydrolysing a meat-based mixture in the presence of an enzyme to form meat-based hydrolysate prior to the fermenting.

The enzyme may be any suitable hydrolysing enzyme. For example, the enzyme may be, but not limited to, a protease.

The meat-based mixture may be formed by mixing a suitable amount of meat by-product and distilled water. For example, the meat-based mixture may be formed by mixing meat by-product and distilled water in a ratio of 1:2 by weight.

5 The hydrolysing may be under any suitable conditions. For example, the hydrolysing may be at a suitable pH and temperature.

In particular, the hydrolysing may be at a pH of 5.5-7.5. Even more in particular, the pH may be about 5.5-6.5. The pH may be adjusted by adding a suitable amount of acid. For example, the acid may be a food-grade acid. In particular, the acid may be lactic acid.

10 In particular, the hydrolysing may be at a temperature of 45-60°C. Even more in particular, the temperature may be about 45-55°C.

The hydrolysing may be for a suitable period of time. For example, the hydrolysing may be for 3-8 hours. In particular, the hydrolysing may be for 3-8 hours, 4-7 hours, 5-6 hours. Even more in particular, the hydrolysing may be for 4-6 hours. The hydrolysing
15 may be terminated by deactivating the enzyme. The deactivating may be by any suitable means.

The process may further comprise heat treating the meat-based seasoning following the fermenting. The heat treating may be under suitable conditions. In particular, the heat treating may be by Maillard reaction.

20 According to a particular aspect, the heat treating may be at a suitable temperature. For example, the heat treating may be at a temperature of 90-121°C. In particular, the heat treating may be at a temperature of about 95-120°C , 98-115°C, 100-112°C , 105-110°C , 106-108°C. Even more in particular, the temperature may be 90-100°C.

The heat treating may be for a suitable period of time. For example, the heat treating
25 may be for 30-120 minutes. In particular, the heat treating may be for about 35-115 minutes, 40-110 minutes, 45-100 minutes, 50-90 minutes, 55-75 minutes, 60-70 minutes. Even more in particular, the heat treating may be for 45-60 minutes.

The heat treating may be performed on the meat-based seasoning at a suitable pH. For example, the meat-based seasoning may be at a pH of 4-6. In particular, the pH may be 5.2-5.5.

5 The heat treating may eliminate any dominant off-flavour compound such as hexanal in the meat-based hydrolysates. The high temperature of the heat treating may promote the generation of volatile compounds which may give a roasted and sweet flavour note to the heat treated meat-based seasoning.

A schematic representation of the process of the present invention is shown in Figure 1. In particular, Figure 1 shows a general process of forming meat-based seasoning
10 comprising: hydrolysing a meat-based mixture to form meat-based hydrolysate; fermenting the meat-based hydrolysate to form a meat-based seasoning; and heat treating the meat-based seasoning.

The hydrolysing, fermenting and heat treating according to a particular embodiment of the present invention are schematically represented in Figures 2, 3 and 4, respectively.

15 The method according to the present invention may be a low-waste method. In other words, the method produces little waste and at the same time upcycles waste meat by-products in preparing the meat-based seasoning. Accordingly, the method of the present invention overcomes the problem of meat by-product wastage and reduces food wastage, and additionally, forms a value-added and functional food product. The
20 method is also simple and does not involve the use of expensive solvents, making it easier to scale-up the method.

Further, the method results in the reduction of fermentation time as compared to prior art methods which may sometimes involve months of fermentation. In particular, the non-halophilic microorganism fermentation requires minimal chemicals, reaction time
25 and also generates little side products.

According to a second aspect, the present invention provides a meat-based seasoning prepared from the process of the first aspect.

According to a third aspect, there is also provided a meat-based seasoning comprising 10-45 mg/mL free amino acid. For example, the meat-based seasoning may comprise
30 15-40 mg/mL, 20-35 mg/mL, 22-30 mg/mL, 25-28 mg/mL free amino acid. In particular,

the meat-based seasoning may comprise 30-40 mg/mL free amino acid. Even more in particular, the meat-based seasoning may comprise 35 mg/mL free amino acid.

For the purposes of the present invention, free amino acids are defined as single amino acids, which need no digestion and are more available for absorption by the body.

5 Further, free amino acids may be ready for development of proteins.

The meat-based seasoning according to the present invention may have a low sodium content. For example, the sodium content of the meat-based seasoning may be ≤ 2 wt %.

According to a particular aspect, the meat-based seasoning may have a low carbohydrate content. For example, the carbohydrate content of the meat-based seasoning may be ≤ 1 wt %.

In view of the low sodium and/or carbohydrate content, as well as the high free amino acid content, the meat-based seasoning is very healthy and provides good nutrition for human body absorption.

15 Having now generally described the invention, the same will be more readily understood through reference to the following embodiment which is provided by way of illustration, and is not intended to be limiting.

Examples

Example 1 - Production of a meat hydrolysate from pork trimmings

20 The process of enzymatic hydrolysis is shown schematically in Figure 2. Thawed minced raw pork trimmings were suspended (1:2, w/v) with distilled water in Duran® laboratory bottles (Merck, Germany). After homogenizing, the mixture was first placed in a water bath (SW22, Julabo®, Germany) for 30 minutes to reach a desired temperature. The mixture was then adjusted to a desired pH at this temperature using
25 1 M lactic acid (Merck, Germany) or 1 M sodium hydroxide (NaOH) (Merck, Germany) solution. Subsequently, one type of protease was added into each bottle according to a specific enzyme/substrate ratio and homogenized immediately. All the homogenized bottles were then put back to the water bath and incubated at a shaking rate of 200 rpm for 6 hours. Table 1 shows the selection of the incubation factors for each enzyme,
30 which was according to preliminary experimental results.

	Flavourzyme®	Protamex®	Alcalase®	Neutrase®
Temperature (°C)	50 °C	50 °C	30-65 °C	40-50 °C
E/S ratio (w/w)	6.0 %	6.0 %	6.0 %	6.0 %
pH	6.0 – 6.5	6.5	7.0 – 9.0	7.0

Table 1: Conditions for pork trimmings hydrolysis using four proteases

During the hydrolytic process, approximately 3.0 mL of hydrolysate were removed and added into a new tube and immediately put into a water bath at 85 °C for 15 minutes (Nilsang et al., 2005) to terminate the enzyme reaction at time intervals of 0, 1, 2, 3, 4, 5 and 6 hours. The hydrolysate obtained at each time point was analyzed for degree of hydrolysis (DH), and the final hydrolysate product was further determined for protein recovery (PR).

Determination of degree of hydrolysis and protein recovery

The DH of each sample was calculated using the relationship between α -amino nitrogen (AN) and total nitrogen (TN) according to the equation below:

$$DH(\%) = \frac{\alpha - AN}{TN} \times 100 \quad (1)$$

The amount of α -amino nitrogen present in the hydrolysates was determined by modified formaldehyde titration method (Nilsang et al., 2005). The volume of the soluble fraction was recorded and the total protein in the supernatant was determined using the Kjeldahl method PR (%), calculated using the following equation:

$$PR(\%) = \frac{\text{total protein in supernatant (g)}}{\text{total protein in substrate (g)}} \times 100 \quad (2)$$

Experiment design for optimization

The Box-Behnken design was employed to optimize the hydrolysis of pork for the release of amino acids. The parameters and levels determined are shown in Table 2.

Process variable	Coded levels		
	-1	0	1
Temperature, °C (A)	45	50	55
Enzyme to substrate ratio, % (w/w) (B)	3.0	6.0	9.0
pH (C)	5	6	7

Table 2: Values of coded levels used in optimization of enzymatic hydrolysis of pork trimmings

The response surface methodology (RSM) method was applied to identify the optimum levels of the three variables. The three variables studied were temperature (A), E/S ratio (B) and pH (C). DH was selected as the response surface for the combination of the independent variables. To simplify the optimization equation, E/S ratio (B) in Table 2 was calculated as a percentage (enzyme/substrate: w/w). It is assumed that the estimated response surface DH can be described by the following quadratic equation (Dey & Dora, 2014):

$$Y = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_{ii} X_i^2 + \sum_{i=1}^2 \sum_{j=i+1}^3 \beta_{ij} X_i X_j \quad (3)$$

Where Y is the dependent variable (DH), β_0 is constant, β_i , β_{ii} , β_{ij} and are coefficients, estimated by the model. X_i and X_j are levels of the independent variables. They represent the linear, quadratic and cross product effects of the A, B and C factors on the response, respectively. The model evaluated the effect of each independent variable on a response.

Amino acid composition

The free amino acid composition of samples produced by enzymatic hydrolysis was quantified by HPLC performed using a Waters AccO-Tag Nova-Pak C18 column with dimensions of 150 x 3.9 mm, packing material of silica base bonded with C_{18} and having particle size of 4 μ m in diameter. Pre-column derivatisation of amino groups with 6-aminoquinolyl-N hydroxysuccinimidyl carbamate (AQC) was conducted using a Waters AccQ-Tag Ultra Chemistry Kit (Dublin, Ireland). HPLC analysis of the derivatised amino acids was performed.

Evaluation of proteases for enzymatic hydrolysis of pork trimmings

Evolution of DH during hydrolysis, and PR after 6 hours of hydrolysis by four proteases are presented in Figure 5. As shown in Figure 5 (a), after 6 hours of hydrolysis, the pork hydrolyzed with Flavourzyme had the highest DH of 45.49 %, which was significantly ($P < 0.05$) higher than the DH of the other proteases, followed by Alcalase, Protamex and Neutrase with a DH of 25.40 %, 20.12 % and 19.27 %, respectively. Figure 5 (a) also shows that the DH of all enzymes increased with time, but the rate of increases became slower at the later stages of hydrolysis except for Flavourzyme. At

each sampling point, the DH of Flavourzyme was significantly ($P < 0.05$) higher than that of the remaining enzymes during the whole process.

In addition, Figure 5 (b) shows that Flavourzyme achieved the highest PR of 55.87 % after 6 hours of hydrolysis, followed by Protamex, Alcalase, and Neutrase at 55.50 %, 53.00 %, and 51.75 %, respectively. However, there was no significant difference ($P \geq 0.05$) of PR among the four proteases. Flavourzyme was selected to produce amino acids-rich pork hydrolysate using RSM.

Optimization of pork trimmings hydrolysis using RSM

As shown in Figure 5, Flavourzyme is the most suitable protease to obtain the highest DH in pork trimmings hydrolysis. Thus, RSM was applied to this protease and DH was chosen as the response factor in combining the independent variables in Table 3.

Runs	Coded levels			DH (%)
	A	B	C	
1	0	0	0	48.06
2	0	-1	-1	36.34
3	0	0	0	47.04
4	1	-1	0	40.34
5	-1	0	-1	38.90
6	0	-1	1	39.95
7	-1	-1	0	37.83
8	0	0	0	48.08
9	1	0	-1	40.59
10	-1	0	1	39.83
11	0	1	-1	40.87
12	1	1	0	42.55
13	0	1	1	41.51
14	0	0	0	48.79
15	1	0	1	42.99
16	-1	1	0	42.94
17	0	0	0	46.90

Table 3: Coded level combinations of Box-Behnken design matrix and the response of the dependent variable degree of hydrolysis (% DH) for pork trimming hydrolysis by Flavourzyme

Each experimental run was conducted independently with a random order to reduce the systematic errors in the responses. The quadratic model below was generated by analyzing the obtained experimental data.

$$Y = 47.77 + 0.8713A + 1.68B + 0.9475C - 0.725AB + 0.3675AC - 0.7425BC - 2.97A^2 - 3.88B^2 - 4.22C^2 \quad (4)$$

The equation showed a good fit with the data obtained from experiments as the adjusted coefficient of determination (R_{adj}^2) was 0.9644, which suggests this equation can explain 96.44% of the variabilities in this study. Analysis of variance (ANOVA) of the model is shown in Table 4.

Source	Sum of Squares	Degree of Freedom	Mean Square	F-value	P-value
Model	236.52	9	26.28	49.23	< 0.0001
A-Temp	6.07	1	6.07	11.38	0.0119
B-E/S	22.48	1	22.48	42.11	0.0003
C-pH	7.18	1	7.18	13.45	0.0080
AB	2.10	1	2.10	3.94	0.0876
AC	0.5402	1	0.5402	1.01	0.3479
BC	2.21	1	2.21	4.13	0.0816
A ²	37.25	1	37.25	69.78	< 0.0001
B ²	63.53	1	63.53	119.01	< 0.0001
C ²	75.05	1	75.05	140.59	< 0.0001
Residual	3.74	7	0.5338		
Lack of Fit	1.23	3	0.4088	0.6515	0.6225
Pure Error	2.51	4	0.6276		
Total	240.26	16			

Table 4: Analysis of variance for the response of DH

The P (probability) value of the model was significantly low ($P < 0.0001$) with an insignificant ($P = 0.6225$) lack-of fit. The determined coefficient ($R^2 = 0.9844$) was desired, reflecting a low experimental error based on the ANOVA results. The ratio of the enzyme to substrate (B) had the most significant effect in gaining the maximum DH, while temperature (A) and pH (C) also significantly affected the DH. In addition, the quadratic terms (A², B², and C²) had significant effects on the DH.

The optimal conditions adopted was temperature (A) at 50.64 °C, enzyme/substrate ratio (B) at 6.58 (w/w), and pH (C) at 6.10. The maximum DH predicted under this condition is 48.04 %. Moreover, the maximum DH obtained from experiments was 48.79 %, which is very close to the model predicted value. In all, this significantly fitted ($P < 0.001$) model indicated that the enzymatic hydrolysis largely relies on the dosage of the added enzymes, followed by reaction pH and temperature, as seen in Table 4.

Amino acid profile of pork trimmings and hydrolysate

The amino acid composition of freeze-dried raw pork trimmings (0 hour), freeze-dried non-enzyme treated control A (6 hours in water bath but without 85 °C, 15 minutes heat treatment), freeze-dried non-enzyme treated control B (6 hours in water bath with 85 °C, 15 minutes heat treatment) and enzymatic hydrolysate (6 hours) is presented in Table 5.

Nineteen amino acids were determined for the samples. All amino acids increased significantly ($P < 0.05$) after 6 hours of enzymatic hydrolysis, by approximately 20 times compared to the raw samples and non-enzyme treated control samples, while majority of the raw and control samples showed little differences among each other ($P \geq 0.05$). These indicated that Flavourzyme hydrolysis has a positive effect on the acceleration in proteolysis. The results obtained confirm that enzymatic proteolysis can generate a large amount of low molecular weight nitrogenous compounds including peptides and free amino acids. The essential amino acids such as isoleucine + leucine (15.79 %), lysine (9.40 %), valine (5.95 %), phenylalanine (5.78 %) and methionine (4.92 %) made up to 41.84 % of all amino acids detected, even exceeding the reference values (40 %) for infants recommended by FAO/WHO (1990).

As seen in Table 5, the hydrolysate obtained had a high concentration of taste-enhancing amino acids, including glutamic acid (8.44 %), aspartic acid (4.76 %), glycine (1.97 %) and alanine (5.01 %), which made up to 20.18 % of the total amino acids. Accounting for 9.33 % of the total amino acids of the hydrolysate, arginine is classified as a conditionally essential amino acid since it involves the many physiological metabolism including protein synthesis and energy conversion (Morris, 2005). Histidine and glutamine (7.69 %), tryptophan (2.00 %), and methionine (4.92 %) were also reported to exhibit notable antioxidant activities, having synergistic effects with α -tocopherol (Zhang et al., 2017). However, the sulfur-containing amino acids, cystine, accounted for the least percentage (0.57 %) of the total amino acids.

In summary, the effectiveness of four microbial proteases in hydrolysing pork trimmings were investigated, and it was shown that Flavourzyme provides the highest degree of hydrolysis (DH). The DH was significantly affected by the hydrolytic conditions including the enzyme/substrate ratio, temperature, pH and reaction time. The optimal conditions obtained using RSM for Flavourzyme were 6.58 (w/w), 50.64 °C and 6.10 for

ratio of enzyme/substrate, temperature and pH, respectively for 6-hour hydrolysis to yield the maximum DH of 48.04 %. The pork hydrolysate enriched with free amino acids can be processed into food flavorings and/or nutrient supplements, enabling effective valorization of pork by-products such as trimmings in the food industry.

5 Example 2 – Fermentation of meat hydrolysate with yeasts

The fermentation process is shown schematically in Figure 3.

Yeast strains and culture media

Four yeast strains were used. *Candida versatilis* NCYC1433 was purchased from National Collection of Yeast Cultures (Norwich, England); *Torulaspora delbrueckii*
10 Prelude, *Saccharomyces cerevisiae* MERIT.ferm, and *Pichia kluyveri* FrootZen were obtained from Chr. Hansen (Hoersholm, Denmark). Two loopfuls of freeze-dried yeast were transferred to 20 mL of sterile yeast-malt (YM) broth which contained 1 % glucose, 0.5 % bacteriological peptone, 0.3 % yeast extract and 0.3 % malt extract at pH 5.0. The inoculated yeasts were incubated statically at 30 °C for 48 hours. Sterile
15 glycerol (Merck, Singapore) was added into each culture to achieve a final glycerol concentration of 30 wt % and cultures were kept at -80 °C before use.

Amino acids	mg/100g (freeze-dried samples)		
	Raw samples (0 h)	Control A* (6 hours)	Control B** (6 hours)
Asp	18.31 ± 4.45 ^a	20.35 ± 4.11 ^a	19.14 ± 7.34 ^a
Ser Asn	26.84 ± 6.97 ^a	29.18 ± 4.81 ^a	31.15 ± 6.25 ^a
Glu	57.17 ± 10.37 ^a	63.41 ± 6.64 ^a	65.15 ± 13.73 ^a
Gly	28.34 ± 5.65 ^a	27.93 ± 3.67 ^a	30.87 ± 6.61 ^a
His Gln	27.60 ± 6.14 ^{ab}	20.44 ± 2.67 ^a	36.33 ± 6.58 ^b
NH ₃	28.68 ± 5.11 ^a	29.15 ± 2.10 ^a	28.58 ± 6.37 ^a
Arg	343.89 ± 52.58 ^a	356.56 ± 34.15 ^a	462.93 ± 143.36 ^a
Thr	27.09 ± 4.24 ^a	29.15 ± 0.59 ^{ab}	39.33 ± 10.15 ^b
Ala	78.93 ± 12.29 ^a	83.41 ± 4.87 ^a	94.47 ± 20.60 ^a
Pro	28.38 ± 4.09 ^a	36.66 ± 1.78 ^a	32.29 ± 6.46 ^a
Cys	3.05 ± 0.08 ^b	2.99 ± 0.13 ^{ab}	2.73 ± 0.24 ^a
Tyr	8.40 ± 0.51 ^a	9.67 ± 0.36 ^a	12.72 ± 1.73 ^b
Val	22.92 ± 2.91 ^a	26.77 ± 1.37 ^{ab}	31.03 ± 6.05 ^b
Met	14.25 ± 1.57 ^a	15.40 ± 0.47 ^a	16.92 ± 2.79 ^a
Lys	8.87 ± 0.81 ^b	5.90 ± 0.50 ^a	8.63 ± 0.87 ^b
Ile	14.39 ± 1.65 ^a	17.84 ± 0.85 ^{ab}	20.11 ± 3.53 ^b
Leu	22.06 ± 2.99 ^a	29.33 ± 1.81 ^{ab}	34.09 ± 6.53 ^b
Phe	15.32 ± 1.43 ^a	20.58 ± 0.79 ^b	23.12 ± 4.14 ^b
Trp	4.73 ± 0.51 ^a	5.48 ± 0.29 ^{ab}	6.50 ± 1.24 ^b
Total	779.23 ± 132.32^a	830.20 ± 41.46^a	996.08 ± 271.27^a
			17095.09 ± 2013.07^b

^{a,b,c} Different letters in the same row indicate significant differences at $p \leq 0.05$. Values are mean $\pm$ standard deviation ($n = 5$).

*Control A: non-enzymatic treated samples without 85 °C, 15 min heat treatment at the end of hydrolysis.

** Control B: non-enzymatic treated samples with 85 °C, 15 min heat treatment at the end of hydrolysis.

5 **Table 5: Amino acid composition of raw pork trimmings, non-enzyme treated controls A and B, enzymatic hydrolysate**

Fermentation of meat hydrolysate with yeasts

Glucose (D-(+)-glucose monohydrate, $\geq 99.0\%$, Merck, Singapore) was added to the thawed meat hydrolysate at a ratio of 2 % (w/w), and the final pH of meat hydrolysate was adjusted to 4.5 ± 0.05 using 1 M of lactic acid. The mixture was then pasteurized
5 at 85 °C for 15 minutes and cooled to room temperature and the effectiveness of pasteurization was verified by plating prior to inoculation.

Thawed yeasts cultures were transferred to the YM broth at 10 % (v/v) and incubated statically at 30 °C for 48 hours. Another sub-culturing was conducted using the same method and the obtained yeast culture was centrifuged at $8000 \times g$ at 4 °C for 5
10 minutes and the pellet was washed twice using 5 mL of 0.85 wt % saline solution.

Subsequently, each yeast pellet was re-suspended in pasteurised meat hydrolysate to adjust to an appropriate cell concentration ($10^5 - 10^6$ CFU/mL). Three mL of each re-suspended yeast culture was then added to the 30 mL of pasteurised meat hydrolysate to obtain a final inoculated cell count of 10^5 CFU/mL and thoroughly mixed. The
15 inoculated meat hydrolysate samples were incubated at 30 °C for 15 days statically with periodic samplings. Uninoculated pasteurised meat hydrolysate was incubated under the same condition to serve as the control. The samples taken were kept at -20 °C before analysis.

Yeast enumeration and pH measurement

20 Cell enumeration was performed by serial dilution in 0.1 % (w/v) sterile peptone water (Oxoid, Basingstoke, UK), followed by spread plating onto potato dextrose agars (Oxoid, Basingstoke, UK). All plates were incubated at 30 °C for 48 hours, except for strain 1433 which was incubated for 5 days. The pH was measured with a pH meter (Metrohm, Herisau, Switzerland).

25 Analysis of total reducing sugar and organic acids

Total reducing sugar contents of control and fermented samples were determined using the 3,5-dinitrosalicylic acid (DNS) method (Wood et al., 2012) with some modifications. 10 μ L of a fermented sample and 190 μ L of the DNS reagent were mixed evenly in a centrifuge tube (Merck, Singapore). Thereafter, the tube was heated

for 5 minutes in a boiling water bath and cooled in an ice box. 100 µL of a reacted sample was pipetted to a 96-microtiter plate and read at 540 nm.

Organic acid extracts of fermented meat sauces were prepared using a method described in Toh and Liu (2017). An aliquot of 0.1 % (v/v) H₂SO₄ was added to a sample at a ratio of 1:1 (v/v) in a centrifuge tube and stored at 4 °C for 1 hour to allow protein precipitation. The tubes were centrifuged at 10,000 × *g* at 4 °C for 10 minutes and the supernatant was collected after filtering through a 0.2-µm Minisart RC 15 syringe filter (Sartorius, Goettingen, Germany). Organic acids analysis by high performance liquid chromatography (HPLC) was performed using a Supelcogel C-610 H column (Supelco, Bellefonte, PA, USA). The mobile phase was 0.1 % (v/v) H₂SO₄, and was set at a flow rate of 0.4 mL/min at 40 °C (Lee et. al., 2013). Organic acid compounds were detected at 210 nm in an SPD-M20A photodiode array detector (Shimadzu, Kyoto, Japan), and quantified with external standards.

Analysis of free amino acids

Free amino acids of fermented meat sauces were extracted using acrylonitrile (ACN) (Merck, Singapore) to precipitate the proteins; 600 µL of ACN, 200 µL of distilled water and 200 µL of a sample were mixed in a centrifuge tube and stored at 4°C for at least 1 hour. The samples were centrifuged at 10,000 × *g* at 4°C for 10 minutes and the supernatant was collected after filtering through a 0.2-µm Minisart RC 15 syringe filter (Sartorius, Goettingen, Germany). Pre-column derivatisation of amino groups with 6-aminoquinolyl-N hydroxysuccinimidyl carbamate (AQC) was conducted using a Waters AccQ-Tag Ultra Chemistry Kit (Dublin, Ireland). HPLC analysis of the derivatised amino acids was performed. An amino acid standards mixture (ThermoFisher Scientific, Waltham, MA, USA) was used for identification and quantification.

Analysis of volatile compounds

Extraction and analysis of volatile compounds in fermented meat sauces were conducted using a modified protocol (Vong and Liu, 2017). Prior to extraction, samples were thawed and heat-treated in a water bath at 60 °C for 2 minutes. The volatile compounds were extracted by headspace-solid phase microextraction (HS-SPME), coupled with gas chromatography–mass spectrometry and semi-quantified by flame ionisation detector (GC-MS/FID).

5 mL of samples were added to a 20-mL glass vial with a polytetrafluoroethylene (PTFE) septum. An 85- μ m carboxen/polydimethylsiloxane SPME fiber (CAR/PDMS, Supelco, Bellefonte, PA, USA) was used to extract the headspace volatile organic compounds at 60 °C for 30 minutes under 250 rpm agitation using a Combi Pal autosampler (CTC Analytics, Zwingen, Switzerland). The extracted compounds were separated on a DB-FFAP capillary column (60 m length, 0.25 mm internal diameter, 0.25 μ m film thickness, Agilent, Santa Clara, CA, USA) with helium as the carrier gas at a flow rate of 1.2 mL/min (Agilent 5975C triple-axis MS and FID). The conditions of separation (Gao et al., 2016) were: oven initial temperature set at 40 °C for 3 minutes, then increased to 90 °C at a rate of 5 °C per minute without holding, and increased to 230 °C at a rate of 10 °C per minute with holding time of 7 minutes.

The identification of compounds was based on the comparison of their mass spectra with a database in NIST 8.0 and Wiley 275 MS libraries. Verification of compounds was based on their linear retention index (LRI) values. The LRI value of each compound was calculated using its retention time. GC-FID peak areas were used to semi-quantify the volatiles and the relative peak area (RPA) expressed in percentage was calculated in each major group of compounds.

Statistical analysis

Free amino acids of fermented meat sauces were extracted using acrylonitrile (ACN) (Merck, Singapore) to precipitate the proteins. 600 μ L of ACN, 200 μ L of distilled water and 200 μ L of a sample were mixed in a centrifuge tube and stored at 4 °C for at least 1 hour. The samples were centrifuged at 10,000 $\times g$ at 4 °C for 10 minutes and the supernatant was collected after filtering through a 0.2- μ m Minisart RC 15 syringe filter (Sartorius, Goettingen, Germany).

Pre-column derivatisation of amino groups with 6-aminoquinolyl-N hydroxysuccinimidyl carbamate (AQC) was conducted using a Waters AccQ-Tag Ultra Chemistry Kit (Dublin, Ireland). HPLC analysis of the derivatised amino acids was performed. An amino acid standards mixture (ThermoFisher Scientific, Waltham, MA, USA) was used for identification and quantification.

Yeast populations and pH changes

- As shown in Figure 6 (a), growth pattern was similar for wine yeast strains Prelude, Merit, and FrootZen, where the total cell counts respectively increased from approximately 6.0 to 7.71, 7.64 and 7.34 log CFU/mL by Day 1. Thereafter, the cell counts respectively dropped gradually to 5.76, 6.59 and 5.78 log CFU/mL by Day 15. In contrast, the cell counts of soy sauce yeast 1433 significantly ($P < 0.05$) increased from 5.45 to 7.22 log CFU/mL only by Day 3 and remained relatively stable until Day 5 when the cell counts decreased to 6.10 log CFU/mL. These results showed that the pork hydrolysate contained a sufficient amount of yeast assimilable nitrogen and micronutrients to support yeast growth.
- The time course of pH changes in the meat sauce during fermentation is shown Figure 6 (b). The pH values of all samples decreased slightly but varied between 4.40 to 4.50 throughout the fermentation. This was a result of production of small amounts of acids, as seen in Figure 7, and CO₂.

Total reducing sugar and organic acids

- Changes of total reducing sugar (mainly glucose) and organic acids during meat sauce fermentation are presented in Figure 7. As seen in Figure 7 (a), total reducing sugar in all yeast fermented samples continually decreased from 24.38 mg/mL (Day 0) to 1.0 mg/mL (Day 15). In the Examples, the total reducing sugar in meat sauce samples was mainly glucose, which served as the major carbon and energy source of yeasts. Sugar consumption was a reflection of yeast growth rate and magnitude. Sugar consumption results correlated well with the yeast cell count data shown in Figure 6 (a), where the wine yeasts were actively consuming the sugar and grew most from Day 0 to Day 1, whereas in the case of the soy sauce yeast, the major sugar consumption occurred from Day 1 to 5 of the exponential phase.
- The organic acid changes of all samples are illustrated in Figures 7 (b) to (g). Citric and lactic acids showed no significant changes ($P > 0.05$) (Figures 7 (b) and (f)), while malic acid was produced by Day 1 and remained stable (Figure 7 (c)). The four yeast strains presented similar trends of producing pyruvic acid (Figure 7 (d)) and acetic acid (Figure 7 (g)) during fermentation, and acetic acid production by the soy sauce yeast was notably high. Succinic acid either increased or decreased, depending on yeast (Figure 7 (e)).

The consistent pyruvic acid production might reflect the continually fermenting process of yeasts. Acetyl coenzyme A (acetyl-CoA) derived from pyruvate may have been enzymatically oxidized into acetic acid in order to regenerate nicotinamide adenine dinucleotide (NAD) + hydrogen (H) (NADH) and balance redox, instead of being reduced to ethanol. As seen in Figures 7 and 8, production of acetic acid and ethanol by the four yeasts seemed to be inversely correlated. *C. versatilis* and *P. kluyveri* are known to be more oxidative and thus, producing more acetic acid. In contrast, *S. cerevisiae* and *T. delbrueckii* are more fermentative and produce more ethanol. Citric, malic and succinic acids are intermediates of the tricarboxylic acid cycle (TCA cycle) and their production during fermentation could be the result of the physiological and redox state of the yeast, though there was no change in citric acid. In all, the production of organic acids, especially acetic acid, might affect the sensory properties of the fermented meat sauce.

Free amino acids profile

Amino acids are not only the major determinants of the taste attributes of the fermented meat sauce but also the precursors of aroma compounds produced by yeasts. As such, the amino acid profiles of different samples were determined and compared. Table 6 shows the changes in amino acid concentrations of control and samples fermented with four yeast strains. The total free amino acid concentrations of fermented meat sauces significantly ($P < 0.05$) decreased after fermentation for 15 days. The total amino acids of the meat sauce fermented by strain Merit decreased the most, almost by two-thirds, followed by strains Prelude (50%) and FrootZen (45 %), respectively, whereas strain 1433 decreased the least (30%).

The sweet tasting amino acids such as glycine (Gly) and alanine (Ala) accounted for around 6% of the total amino acids in the control, while bitter tasting amino acids including valine (Val) and leucine (Leu) accounted for nearly 17%. After fermentation, the percentages of these amino acids remained nearly unchanged for all yeasts. In addition, aspartic acid (Asp) and glutamate (Glu), which contribute to sour and umami tastes, accounted for approximately 13% in the control and all the fermented meat sauces. In traditional cooked meat sauces, sulfur-containing compounds are particularly important in contributing to the "roast-meaty" aroma. A major route of producing these meat-like flavor compounds is the Maillard reaction between the

sulfur-containing amino acid such as cysteine (Cys) and methionine (Met) and reducing sugars. Cys was found in low concentrations in the control sample (5.09 mg/L) and decreased by almost half after 15 days of fermentation with all yeasts. The amount of Met was four times higher than Cys (21.63 mg/L) and was utilized by 50% by the wine yeasts, whereas the soy sauce yeast 1433 used only about 33% of Met.

Volatile compounds

A total of 17 and 34, 30, 37, 30 volatile compounds were respectively identified in the control and meat sauces fermented by yeasts 1433, Prelude, Merit and FrootZen, on Day 15. The volatile compounds are classified into seven groups: acids, ethanol, alcohols (excluding ethanol), aldehydes, ketones, esters and furans, as illustrated in Figure 8.

The main compounds identified in the control belonged to aldehydes and furans groups, with RPA of 67.11% and 24.21%, respectively (Figures 8 (d) and (g)). The dominant compounds in these two groups were respectively hexanal and 2-pentylfuran. Hexanal is frequently linked to green, herbaceous and beany flavors associated with lipid oxidation, and is often determined as the onset of rancidity. The dominance of hexanal and other aliphatic aldehydes such as pentanal, heptanal, octanal and nonanal was likely related to the oxidation of unsaturated lipids in meat which would have happened in the 6-hour enzymatic hydrolysis. 2-pentylfuran, however, was likely to be naturally present in the raw pork, since it is known to be produced by mammalian metabolism. The dominance of hexanal and 2-pentylfuran likely imparted an overall undesirable grassy and "cardboard-like" flavor to the non-fermented pork trimming hydrolysate.

Amino acids	Control (Day 0)	Fermented meat sauce (Day 15)		
		1433	Prelude	Merit
Asp	19.07 ± 2.34 ^d	14.16 ± 1.44 ^c	9.08 ± 0.71 ^{ab}	6.38 ± 0.33 ^a
Ser and Asn	37.98 ± 4.66 ^c	26.42 ± 3.87 ^b	19.48 ± 2.29 ^b	12.28 ± 1.81 ^a
Glu	39.06 ± 2.50 ^d	27.06 ± 3.32 ^c	21.00 ± 2.41 ^b	13.20 ± 0.10 ^a
Gly	8.41 ± 0.59 ^d	6.42 ± 0.26 ^c	3.80 ± 0.04 ^a	3.99 ± 0.33 ^{ab}
His and Gln	27.05 ± 3.54 ^c	14.78 ± 0.93 ^b	9.26 ± 0.89 ^a	8.50 ± 1.14 ^a
NH ₃	2.91 ± 0.27 ^b	2.69 ± 0.29 ^b	2.14 ± 0.19 ^a	1.68 ± 0.18 ^a
Arg	55.66 ± 8.86 ^c	34.80 ± 4.45 ^b	25.01 ± 1.57 ^{ab}	15.41 ± 1.38 ^a
Thr	25.41 ± 3.34 ^d	17.00 ± 1.92 ^c	12.12 ± 1.19 ^a	7.51 ± 0.42 ^a
Ala	22.44 ± 1.79 ^d	16.79 ± 1.17 ^c	12.51 ± 1.22 ^b	8.36 ± 0.97 ^a
Pro	6.38 ± 0.60 ^d	5.70 ± 0.31 ^{cd}	4.04 ± 0.05 ^a	4.46 ± 0.37 ^{ab}
Cys	5.09 ± 0.54 ^c	3.75 ± 0.34 ^b	2.52 ± 0.16 ^a	2.94 ± 0.03 ^a
Tyr	8.52 ± 1.42 ^b	5.16 ± 0.04 ^a	3.42 ± 0.61 ^a	3.56 ± 0.41 ^a
Val	29.38 ± 3.30 ^d	21.18 ± 0.61 ^c	14.70 ± 1.21 ^{ab}	10.66 ± 1.27 ^a
Met	21.63 ± 3.25 ^c	15.94 ± 2.06 ^b	10.73 ± 0.91 ^a	8.00 ± 1.02 ^a
Lys	41.71 ± 4.87 ^d	28.92 ± 4.11 ^c	20.29 ± 2.82 ^{ab}	15.54 ± 1.53 ^a
Ile	25.12 ± 2.34 ^d	17.76 ± 0.65 ^c	12.97 ± 0.77 ^{ab}	10.89 ± 0.35 ^a
Leu	49.18 ± 6.05 ^c	31.86 ± 4.70 ^b	25.10 ± 2.44 ^{ab}	18.97 ± 2.78 ^a
Phe	27.46 ± 4.28 ^c	18.91 ± 1.26 ^b	13.73 ± 0.83 ^a	11.44 ± 1.46 ^a
Trp	6.29 ± 0.35 ^c	4.57 ± 0.41 ^b	2.78 ± 0.27 ^a	2.88 ± 0.22 ^a
Total	458.77 ± 54.92 ^d	313.87 ± 32.15 ^c	224.69 ± 20.58 ^{ab}	166.65 ± 16.11 ^a

^{a,b,c,d} Different letters in the same row indicate significant differences at $P \leq 0.05$. Values are mean ± standard deviation (n = 5).

Table 6: Free amino acid contents in control and yeast-fermented pork trimmings hydrolysate (mg/L)

Prominent differences were observed in the volatile profiles among meat sauces fermented with different yeasts. In general, ethanol and volatile compounds belonging to volatile acids, other alcohols and esters significantly ($P < 0.05$) increased, whereas aldehydes and furans significantly ($P < 0.05$) decreased after fermentation, as seen in Figure 8. During the catabolism of amino acids, α -keto acids were produced, then decarboxylated to form aldehydes, followed by reduction to alcohols and/or oxidation to carboxylic acids, although only isovaleric acid was produced by the soy sauce yeast. The increases of esters are due to the reaction between alcohols and activated acids (acyl Co-As). The decreases of furans were mainly associated with the reduction of 2-pentylfuran compounds.

A large number of esters were produced, mainly acetyl and fatty acyl esters of ethanol, branched-chain and aliphatic alcohols. As seen in Figure 8, among the four yeast strains, strain FrootZen produced the highest amount of esters whereas strain 1433 produced the least amount. Dominant esters produced by strain FrootZen included isoamyl acetate, isobutyl acetate and 2-phenylethyl acetate, whereas these esters were detected in minor amounts or not produced by strain 1433. These esters were enzymatically produced by the condensation between an alcohol and acetyl-CoA or fatty acyl-CoA catalyzed by alcohol acetyl Co-A or fatty acyl transferases in yeasts, imparting generally fruity flavor notes. The strong banana-like and floral odor produced by strain FrootZen distinguished itself from other yeasts.

Several ketones were produced by some of the yeasts and were likely the product of fatty acid metabolism by the yeasts via β -oxidation. Strain 1433 produced the highest amount of ketones, while ketones were not produced by strain FrootZen. Acetone was the major ketone and only produced by strain 1433.

In summary, one soy sauce yeast (*C. versatilis*) and three wine yeasts (*S. cerevisiae*, *T. delbrueckii*, and *P. kluyveri*) were used to ferment pork trimmings hydrolysate. All yeasts grew well and utilized glucose and free amino acids from the pork trimming hydrolysate to produce pleasant fruity and ester compounds. Undesirable off-odourants aldehydes produced from lipid oxidations were mostly reduced to alcohols. In particular, *P. kluyveri* FrootZen produced a significantly higher amount of esters and presented a very strong "banana-like" odor, whereas *C. versatilis* 1433 produced more

ketone compounds. These findings demonstrate that yeasts are effective transformers of meat hydrolysates for the production of novel seasonings.

Example 3 – Fermentation of meat hydrolysate with lactic acid bacteria (LAB)

The fermentation process is shown schematically in Figure 3.

5 *Bacterial cultures and media*

Six lactic acid bacteria (LAB) strains were used in the present study: *Lactobacillus fermentum* PCC and *Lb. rhamnosus* GG were obtained from Chr. Hansen (Hoersholm, Denmark); *Lb. plantarum* 299V was obtained from Probi (Lund, Sweden); *Lactococcus lactis* subsp. *cremoris* DSM20069 was purchased from DSMZ (Braunschweig, Germany); *Lb. acidophilus* NCFM was attained from Danisco (Copenhagen, Denmark);
10 *Lb. casei* Shirota was isolated from Yakult probiotic cultured milk drink (Yakult Honsha, Japan).

Two loopfuls of freeze-dried LAB cultures were transferred to 20 mL of sterile De Man, Rogosa and Sharpe (MRS) broth (Oxoid, Basingstoke, UK) and incubated statically at
15 37 °C for 24 hours. The cultures were preserved in 30 % sterile glycerol (Merck, Singapore) at -80 °C before use.

Fermentation of meat hydrolysate with LAB

Glucose (2 % (w/w) of D-(+)-glucose monohydrate) ($\geq 99.0\%$, Merck, Singapore) was added to meat hydrolysate, followed by pasteurization at 85 °C for 15 minutes. The
20 effectiveness of pasteurization was verified by plating. Each LAB culture was activated once and sub-cultured once in MRS broth at a ratio of 10 % (v/v) and was incubated at 37 °C for 24 hours. The bacterial pellets were obtained by washing twice with 5 mL of 0.85 wt % saline solution after centrifugation ($8000 \times g$ for 5 minutes, 4 °C).

The bacterial pellets were re-suspended in 5 mL of pasteurized meat hydrolysate to
25 adjust to a desired cell concentration of 10^7 CFU/mL. Three mL of each bacterial suspension was inoculated into 30 mL of meat hydrolysate and thoroughly mixed. The inoculated meat hydrolysates were incubated at 37 °C for 4 days under static conditions with sampling every 24 hours. Uninoculated meat hydrolysate was treated

under the same condition as the control. The fermented samples were kept at -20 °C before analysis.

Bacterial enumeration and pH measurement

Cell enumeration was performed by serial dilution in 0.1 % (w/v) sterile peptone water (Oxoid, Basingstoke, UK), followed by spread plating onto potato dextrose agars (Oxoid, Basingstoke, UK). All plates were incubated at 30 °C for 48 hours. The pH was measured with a pH meter (Metrohm, Herisau, Switzerland).

Analysis of total reducing sugar and organic acids

Total reducing sugar contents of control and fermented samples were determined using the 3,5-dinitrosalicylic acid (DNS) method (Wood et al., 2012) with some modifications. 10 µL of a fermented sample and 190 µL of the DNS reagent were mixed evenly in a centrifuge tube (Merck, Singapore). Thereafter, the tube was heated for 5 minutes in a boiling water bath and cooled in an ice box. 100 µL of a reacted sample was pipetted to a 96-microtiter plate and read at 540 nm.

Organic acid extracts of fermented meat sauces were prepared using a method described in Toh and Liu (2017). An aliquot of 0.1 % (v/v) H₂SO₄ was added to a sample at a ratio of 1:1 (v/v) in a centrifuge tube and stored at 4 °C for 1 hour to allow protein precipitation. The tubes were centrifuged at 10,000 × g at 4 °C for 10 minutes and the supernatant was collected after filtering through a 0.2-µm Minisart RC 15 syringe filter (Sartorius, Goettingen, Germany). Organic acids analysis by high performance liquid chromatography (HPLC) was performed using a Supelcogel C-610 H column (Supelco, Bellefonte, PA, USA). The mobile phase was 0.1 % (v/v) H₂SO₄, and was set at a flow rate of 0.4 mL/min at 40 °C (Lee et. al., 2013). Organic acid compounds were detected at 210 nm in an SPD-M20A photodiode array detector (Shimadzu, Kyoto, Japan), and quantified with external standards.

Analysis of free amino acids

Free amino acids of fermented meat sauces were extracted using acrylonitrile (ACN) (Merck, Singapore) to precipitate the proteins; 600 µL of ACN, 200 µL of distilled water and 200 µL of a sample were mixed in a centrifuge tube and stored at 4 °C for at least 1 hour. The samples were centrifuged at 10,000 × g at 4 °C for 10 minutes and the

supernatant was collected after filtering through a 0.2- μ m Minisart RC 15 syringe filter (Sartorius, Goettingen, Germany). Pre-column derivatisation of amino groups with AQC was conducted using a Waters AccQ-Tag Ultra Chemistry Kit (Dublin, Ireland). HPLC analysis of the derivatised amino acids was performed. An amino acid standards mixture (ThermoFisher Scientific, Waltham, MA, USA) was used for identification and quantification.

Analysis of volatile compounds

Extraction and analysis of volatile compounds in fermented meat sauces were conducted using a modified protocol (Vong and Liu, 2017). Prior to extraction, samples were thawed and heat-treated in a water bath at 60 °C for 2 minutes. The volatile compounds were extracted by HS-SPME, coupled with GC-MS/FID.

5 mL of samples were added to a 20-mL glass vial with a PTFE septum. An 85- μ m carboxen/polydimethylsiloxane SPME fiber (CAR/PDMS, Supelco, Bellefonte, PA, USA) was used to extract the headspace volatile organic compounds at 60 °C for 30 minutes under 250 rpm agitation using a Combi Pal autosampler (CTC Analytics, Zwingen, Switzerland). The extracted compounds were separated on a DB-FFAP capillary column (60 m length, 0.25 mm internal diameter, 0.25 μ m film thickness, Agilent, Santa Clara, CA, USA) with helium as the carrier gas at a flow rate of 1.2 mL/min (Agilent 5975C triple-axis MS and FID). The conditions of separation (Gao et al., 2016) were: oven initial temperature set at 40 °C for 3 minutes, then increased to 90 °C at a rate of 5 °C per minute without holding, and increased to 230 °C at a rate of 10 °C per minute with holding time of 7 minutes.

The identification of compounds was based on the comparison of their mass spectra with a database in NIST 8.0 and Wiley 275 MS libraries. Verification of compounds was based on their LRI values. The LRI value of each compound was calculated using its retention time. GC-FID peak areas were used to semi-quantify the volatiles and the relative peak area (RPA) expressed in percentage was calculated in each major group of compounds.

Statistical analysis

Free amino acids of fermented meat sauces were extracted using acrylonitrile (ACN) (Merck, Singapore) to precipitate the proteins. 600 µL of ACN, 200 µL of distilled water and 200 µL of a sample were mixed in a centrifuge tube and stored at 4 °C for at least 1 hour. The samples were centrifuged at $10,000 \times g$ at 4 °C for 10 minutes and the supernatant was collected after filtering through a 0.2-µm Minisart RC 15 syringe filter (Sartorius, Goettingen, Germany).

Pre-column derivatisation of amino groups with AQC was conducted using a Waters AccQ-Tag Ultra Chemistry Kit (Dublin, Ireland). HPLC analysis of the derivatised amino acids was performed. An amino acid standards mixture (ThermoFisher Scientific, Waltham, MA, USA) was used for identification and quantification.

LAB growth and pH changes

All LAB grew well in the pork trimmings hydrolysate and increased cell counts by 1.5-2.0 log CFU/mL, as illustrated in Figure 9. Figure 9 (a) shows a similar growth trend for strains GG and Shirota. The cell numbers of these two strains respectively increased from 7.06 and 7.39 to 8.64 and 9.24 log CFU/mL in 24 hours and remained stable until the end of fermentation. On the other hand, the cell counts of strains PCC, 299V, DSM20069 and NCFM increased from approximately 7.50 to 8.8 CFU/mL in 24 hours, and gradually fell to around 7.3 log CFU/mL by Day 4.

As shown in Figure 9 (b), the pH of all LAB-fermented hydrolysates dropped significantly ($P < 0.05$) in 24 hours, reaching approximately 3.8 by Day 4 except strain PCC, which reduced pH to only 4.3.

Changes in total reducing sugar and organic acids

As shown in Figure 10 (a), the total reducing sugar contents of all LAB-fermented hydrolysates significantly ($P < 0.05$) decreased after 4 days, although the rate of sugar utilization varied with LAB. Strain PCC consumed the added glucose most rapidly, followed by strains Shirota, GG and NCFM. In particular, strain PCC and Shirota almost used up all the available glucose in the media, reducing glucose from 20.98 mg/mL to only 1.23 and 2.46 mg/mL respectively by Day 4. Significant levels of residual glucose remained at <10 mg/mL in the other LAB-fermented hydrolysates.

The organic acid compositions including citric acid, pyruvic acid, succinic acid, lactic acid and acetic acid of control and fermented samples are shown in Figures 10 (b) to (f). In general, all LAB strains presented similar patterns of the organic acid changes: succinic acid, lactic acid and acetic acid significantly ($P < 0.05$) increased while citric acid significantly ($P < 0.05$) decreased at Day 4. However, pyruvic acid showed no changes. As expected, all strains showed remarkable increases of lactic acid, from around 2 mg/mL at Day 0 to average 15 mg/mL at Day 4, because lactic acid is the main end-product for both homofermentative and heterofermentative LAB. Being heterofermentative, strain PCC produced the lowest amount of lactic acid but the highest amount of acetic acid from glucose, as discussed above.

Changes in free amino acids

A total of 19 types of amino acids were determined in the unfermented pork trimmings hydrolysate and are presented in Table 7. The total amino acid concentrations of the hydrolysates fermented by the six LAB were significantly ($P < 0.05$) different from each other, either increased or decreased, depending on LAB. The total amino acids of the hydrolysate fermented by strain DSM20069 increased by 1.7-fold at 539.08 mg/L compared to the unfermented control, followed by strains NCFM, PCC and Shirota at 504.83, 363.63 and 335.86 mg/L, respectively. In contrast, the total amino acids in strain 299V-fermented hydrolysate decreased by more than 2.5-fold at 126.05 mg/L, followed by strain GG at 219.14 mg/L.

The final total amino acid concentration was a net balance of consumption and production by LAB, depending on their proteolytic activities and autolysis (release of amino acids). The significant production of amino acids by strains DSM20069 and NCFM indicates their stronger proteolytic activities compared to strains PCC and Shirota. Strain DSM20069 is a dairy lactococcal strain and therefore its higher proteolytic activity was expected. On the other hand, strain NCFM is a probiotic strain of *Lb. acidophilus* originally isolated from the human gastrointestinal tract and therefore its higher proteolytic activity was surprising.

In addition, the differences in LAB autolysis may partially account for the different levels of amino acid formation. On the other hand, the significant reduction of total amino acids by strains 299V and GG suggests their weaker proteolytic activities and/or autolysis coupled with stronger consumption, resulting in decrease of amino acid

levels. Gut-friendly *Lb. plantarum* and *Lb. rhamnosus* were able to catabolize almost all kinds of amino acids to produce ammonia, carboxylic acids (such as acetate, propionate, valerate, isovalerate), organic acids, phenolic compounds and gaseous compounds (such as carbon dioxide, hydrogen, hydrogen sulfide and methane).

- 5 Interestingly, the concentration of arginine in the hydrolysate fermented by strains PCC was significantly ($P < 0.05$) reduced to 10.04 mg/L even though its total amount of amino acids increased. The utilization of arginine by LAB is desirable for both the LAB and taste, as arginine is also known to have an extremely bitter and unpleasant taste. The catabolism of arginine via the arginine deiminase pathway generates adenosine
- 10 triphosphate (ATP) and ammonia that are beneficial for bacterial survival. On the other hand, glutamic acid in the hydrolysate fermented by strain Shirota was significantly ($P < 0.05$) increased from 28.17 to 34.00 mg/L while the total free amino acid concentrations were almost unchanged. Although the production and catabolic pathway of glutamic acid in *L. casei* is unclear, the increased amount of glutamic acid could contribute to an
- 15 “umami” flavor to the meat sauce. Besides, the concentration of tryptophan remained unchanged in the meat sauce fermented by strains DSM20069 and NCFM although the total amino acids significantly increased ($P < 0.05$). This could be due to the utilization of tryptophan by these two strains. However, the concentration of sulfur amino acids, cysteine (Cys) and methionine (Met), which are crucial for producing “meaty” odor after
- 20 the Maillard reaction, only accounted for approximately 0.9 % and 4 % of the total determined amino acids for all unfermented and fermented samples.

Amino acids	Control (Day 0)	Fermented meat sauce (Day 4)				
		PCC	299V	DSM20069	GG	NCFM
Asp	16.41 ± 0.78 ^c	20.56 ± 0.88 ^d	5.36 ± 0.38 ^a	24.34 ± 1.46 ^e	11.76 ± 1.37 ^b	21.56 ± 2.25 ^d
Ser and Asn	26.64 ± 3.88 ^c	34.48 ± 0.99 ^d	7.56 ± 0.39 ^a	40.39 ± 3.26 ^e	17.44 ± 0.67 ^b	39.71 ± 1.46 ^e
Glu	28.17 ± 1.57 ^c	46.41 ± 1.94 ^e	9.66 ± 0.48 ^a	52.47 ± 2.83 ^f	22.14 ± 1.39 ^b	52.77 ± 4.20 ^f
Gly	5.16 ± 0.58 ^b	10.16 ± 0.88 ^d	2.35 ± 0.15 ^a	11.80 ± 0.76 ^e	4.79 ± 0.08 ^b	14.56 ± 0.94 ^f
His and Gln	16.39 ± 1.00 ^c	19.28 ± 2.01 ^c	4.52 ± 0.34 ^a	25.89 ± 2.49 ^d	10.07 ± 1.28 ^b	24.81 ± 2.08 ^d
NH ₃	2.19 ± 0.20 ^a	9.38 ± 0.81 ^d	1.78 ± 0.10 ^a	4.97 ± 0.47 ^c	1.89 ± 0.24 ^a	4.18 ± 0.41 ^{bc}
Arg	32.68 ± 4.63 ^c	10.04 ± 0.76 ^a	11.17 ± 0.20 ^a	59.74 ± 2.94 ^d	20.61 ± 2.78 ^b	55.92 ± 3.04 ^d
Thr	17.63 ± 0.73 ^c	19.39 ± 0.11 ^c	6.25 ± 0.38 ^a	30.95 ± 2.90 ^d	11.37 ± 0.30 ^b	29.73 ± 1.66 ^d
Ala	16.35 ± 1.44 ^c	23.70 ± 0.67 ^d	7.04 ± 0.65 ^a	29.13 ± 2.15 ^c	11.55 ± 0.75 ^b	30.94 ± 1.11 ^e
Pro	4.44 ± 0.29 ^{bc}	5.33 ± 0.58 ^c	2.62 ± 0.20 ^a	8.23 ± 0.71 ^d	3.49 ± 0.10 ^{ab}	7.77 ± 0.41 ^d
Cys	3.05 ± 0.30 ^c	3.62 ± 0.17 ^d	1.33 ± 0.11 ^a	4.96 ± 0.18 ^e	2.18 ± 0.14 ^b	4.75 ± 0.43 ^e
Tyr	6.97 ± 1.11 ^{bc}	6.19 ± 0.40 ^{bc}	3.14 ± 0.27 ^a	11.37 ± 0.80 ^d	4.98 ± 0.73 ^{ab}	12.05 ± 1.51 ^d
Val	19.57 ± 1.97 ^c	22.91 ± 0.70 ^c	8.62 ± 0.07 ^a	33.65 ± 1.99 ^e	13.75 ± 0.56 ^b	29.38 ± 2.89 ^d
Met	14.86 ± 0.77 ^c	13.89 ± 0.33 ^c	6.55 ± 0.16 ^a	23.46 ± 1.74 ^e	10.66 ± 0.77 ^b	20.22 ± 1.11 ^d
Lys	29.30 ± 3.14 ^c	38.24 ± 1.98 ^d	9.90 ± 0.27 ^a	55.61 ± 2.97 ^f	18.15 ± 2.19 ^b	46.21 ± 6.37 ^e
Ile	18.00 ± 1.69 ^c	20.09 ± 0.40 ^c	9.11 ± 0.14 ^a	31.41 ± 2.58 ^e	12.58 ± 0.25 ^b	28.35 ± 1.93 ^d
Leu	32.88 ± 4.32 ^c	36.17 ± 0.89 ^c	16.34 ± 0.30 ^a	55.55 ± 1.57 ^e	23.23 ± 0.68 ^b	49.68 ± 2.26 ^d
Phe	19.42 ± 1.02 ^c	18.16 ± 0.34 ^c	9.89 ± 0.87 ^a	28.76 ± 3.28 ^d	13.51 ± 1.38 ^b	26.37 ± 1.20 ^d
Trp	6.31 ± 0.81 ^c	5.62 ± 0.10 ^{bc}	2.88 ± 0.09 ^a	6.39 ± 0.40 ^c	4.99 ± 0.61 ^b	5.87 ± 0.31 ^{bc}
Total	316.45 ± 30.24^e	363.63 ± 14.93^d	126.05 ± 5.55^a	539.08 ± 35.51^e	219.14 ± 16.27^b	504.83 ± 35.58^e

a,b,c,d,e,f Different letters in the same row indicate significant differences at $P \leq 0.05$. Values are mean ± standard deviation of three independent replicates (n = 3).

Table 7: Free amino acid contents in unfermented (control) and LAB-fermented pork trimmings hydrolysates (mg/L)

Volatile compounds

A total of 19, 16, 20, 19, 20, 23 and 17 volatile compounds were respectively identified in the control and meat sauces fermented by strains PCC, 299V, DSM20069, GG, NCFM and Shirota, on Day 4. The identified compounds were separated into five
5 groups: acids, alcohols, aldehydes, ketones, and furans, and the subtotal peak areas (10^6) of each group are summarized in Figure 11.

The major volatile flavor compounds detected in the control were mainly aliphatic aldehydes and furans, accounting for 80.90 % and 10.42 % RPA respectively (Figures 11 (c) and (e)). In particular, hexanal alone accounted for 57.22 % of the total volatile
10 compounds detected in the control. The aliphatic aldehydes that give off a rancid odor likely arose from the oxidation of pork lipids during the 6-hour hydrolysis and furans stemmed from the heat treatment applied prior to fermentation.

In all fermented hydrolysates, total aldehydes decreased to low levels, whereas total acids, alcohols and ketone levels increased substantially ($P < 0.05$). Total furan levels
15 increased by 24 hours, then decreased over time to levels below the initial values, except strain PCC that consumed furans (Figure 11). It was likely that the alcohols (except for ethanol for strain PCC) were derived from the enzymatic reduction of aldehydes and the carboxylic acids (except acetic acid for strain PCC) were formed from the chemical and enzymatic oxidation of aldehydes. However, there was no
20 distinct relationship between the heterofermentative LAB strain PCC and other homofermentative LAB in terms of formation of alcohols and carboxylic acids, suggesting that both groups of LAB possess similar levels of alcohol and aldehyde dehydrogenases activities. Total ketones were largely contributed by 2,3-butanedione (diacetyl) and acetoin generated from citric acid catabolism discussed above. There
25 were little differences in aldehyde reduction among the six LAB. However, there were remarkable distinctions in the formation of total carboxylic acids, total alcohols, total furans (except strain PCC) and total ketones among the LAB with strain NCFM producing the least amount of total alcohols and strain PCC forming the least quantum of total ketones. On the other hand, strain PCC produced the highest amount of total
30 alcohols (mainly ethanol) and strain NCFM formed the highest level of total ketones (mainly diacetyl and acetoin).

There were very few volatiles that originated from amino acids, except 3-methylbutanal and benzaldehyde, both of which could be formed chemically and/or biologically. There were obvious large differences in the formation or degradation of these volatiles among the LAB. For example, only strain GG produced both 2,3-butanedione and acetoin; strains DSM20069 and 299V produced acetoin only, although all LAB degraded citric acid.

In summary, five probiotic LAB and one lactococcal LAB were used to ferment pork trimmings hydrolysates. All LAB grew well in the hydrolysates supplemented with glucose. The major metabolic end-products were lactic acid and acetic acid. Some LAB produced more amino acids than consumed whereas other LAB consumed more than produced. The major lipid-derived aldehydes that would impart an off-odor to the unfermented hydrolysate were largely converted by LAB into corresponding alcohols and acids, effectively improving flavor. However, very little amino acids-derived volatiles were found. The production of lactic acid and acetic acid together with a reduction of aldehydes could lead to the development of a novel liquid pork bioflavoring product.

Example 4 – Fermentation of meat hydrolysate with co-culture of yeast and lactic acid bacteria lactic acid bacteria (LAB)

The fermentation process is shown schematically in Figure 3.

Simultaneous and sequential inoculation of *Lactobacillus fermentum* and *Pichia kluyveri* in pork hydrolysate fermentation were performed, and the microbial viability, physicochemical changes, and volatiles production were investigated.

Five types of inoculation samples were prepared in this experiment: (i) uninoculated pork hydrolysate as control; (ii) pork hydrolysate inoculated with single *L. fermentum* culture; (iii) pork hydrolysate inoculated with single *P. kluyveri* culture; (iv) pork hydrolysate co-inoculated with *L. fermentum* and *P. kluyveri*; (v) pork hydrolysate inoculated with *L. fermentum* first till the pH of media dropped to 4.5, and then *P. kluyveri* was added.

To activate the frozen microbial cultures, *L. fermentum* and *P. kluyveri* stock cultures were transferred to MRS broth (adjusted to pH 5.0) and YM broth at a ratio of 10 %

(v/v) at 30°C for 48 h and 37°C for 24 h, respectively. After centrifugation (8000 × g for 5 min, 4°C) and washing twice with 5 mL of 0.85 wt % saline solution, the cell pellets of each strain were re-suspended in 5 ml of pasteurized pork hydrolysate to make sure the initial inoculum cell counts in pork hydrolysate before fermentation was around 10⁷ for *L. fermentum* and 10⁵ for *P. kluyveri*. The inoculum was added as follows: for single culture inoculation, 0.3 mL of *L. fermentum* or *P. kluyveri* culture suspension were transferred to 30 mL of pork hydrolysate; for co-inoculation, 0.3 mL of *L. fermentum* and 0.3 mL of *P. kluyveri* culture suspension were added to the pork hydrolysate at the start; for sequential inoculation, 0.3 mL of *L. fermentum* culture suspension was inoculated to the pork hydrolysate at day 0 and incubated statically at 37°C for 24 h, after which, 0.3 mL of *P. kluyveri* culture suspension was subsequently added to the media at day 1.

After inoculation, the samples were mixed with gentle shaking and statically incubated at 30°C for 5 days (6 day of fermentation for sequential inoculation). Uninoculated pork hydrolysate was also incubated at 30°C as control. All types of pork hydrolysate were sampled at every 24 h for cell enumeration and pH determination. All samples were then kept at -20°C before other analysis.

Enumeration of yeast or *L. fermentum* was conducted by 10 × diluting samples in 0.1% (w/v) peptone water (Basingstoke, UK), followed by plating on selective agars. Yeast cell counts were determined by spread plating diluted samples onto duplicate potato dextrose agar (PDA, Oxoid, Basingstoke, UK) supplemented with 100 ppm chloramphenicol (Merck, Germany) and incubated plates at 30°C for 48 h, while LAB cell counts were enumerated by pour plating with MRS agar (Oxoid, Basingstoke, UK) containing 500 ppm of natamycin (Natamax®, Danisco A/S, Copenhagen, Denmark) followed by incubation at 37°C for 24 h. The pH changes of all samples were measured directly with a pH meter (Metrohm, Herisau, Switzerland).

Growth of L. fermentum and P. kluyveri in mono- and co-cultures in pork hydrolysate

An antagonistic relationship between *L. fermentum* and *P. kluyveri* was observed during the pork hydrolysate fermentation, regardless of the inoculation sequence. The cell count of *P. kluyveri* significantly ($P < 0.05$) dropped from around 6.80 log CFU/mL at Day 1 to 5.98 and 6.04 log CFU/mL at Day 5, respectively for co-inoculation and

sequential inoculation (Figures 12 (c) and (d)), while cell counts in single *P. kluyveri* culture stayed at 6.45 log CFU/mL at Day 5 after inoculation (Figure 12 (b)). On the other hand, the survival of *L. fermentum* was enhanced in sequentially inoculated cultures, reaching a peak count of 8.82 log CFU/mL at Day 1 and retained the cell count of 8.16 log CFU/mL at Day 6 (Figure 12 (d)). However, similar growth patterns of *L. fermentum* were observed in co-inoculation culture and single culture, where their cell counts respectively decreased from around 8.80 log CFU/mL to a final count of 7.55 and 7.72 log CFU/mL (Figures 12 (a) and (c)).

Glucose and organic acid changes in pork hydrolysate fermentation

The glucose content in all fermented samples decreased to undetectable levels after 5 days of inoculation (Figure 13 (a)). These sugar depletions are expected as *L. fermentum* and *P. kluyveri* utilize glucose for propagation and production of ethanol and/or lactic acid, acetic acid.

For single culture inoculation, *L. fermentum* showed its highest glucose consumption rate of 11.19 mg/mL per day at Day 1, while it was 18.12 mg/mL per day at Day 2 for *P. kluyveri*. In the mixed-inoculation models, less than 1.0 mg/mL of residual glucose were found in co-inoculation at Day 2, whereas it was at Day 4 in sequential inoculation (3 days after the inoculation of *P. kluyveri*).

Free amino acid changes in pork hydrolysate fermentation

The amino acid profile of different inoculation methods was determined and compared in Table 8. Most of the free amino acids in all samples were generally constant with slight fluctuations after fermentation for 5 days. No significant differences ($P \geq 0.05$) were observed in the amino acid utilization between co-inoculation and sequential inoculation. In general, single *P. kluyveri* consumed the most amount of the amino acids and produced a large range of volatile compounds in the pork hydrolysate. *L. fermentum* showed proteolytic activity and thereby increased the amount of total amino acids, though these effects were not statistically significant ($P \geq 0.05$).

Amino acids	Control (Day 0)	Fermented meat hydrolysate			
		<i>L. fermentum</i>	<i>P. kluyveri</i>	Co-inoculation	Sequential inoculation
Asp	11.76 ± 3.86 ^{ab}	16.36 ± 1.14 ^b	10.48 ± 1.83 ^a	15.19 ± 0.58 ^b	13.53 ± 2.33 ^{ab}
Ser and Asn	26.61 ± 0.69 ^a	27.52 ± 0.46 ^a	24.02 ± 3.40 ^a	27.44 ± 1.08 ^a	25.38 ± 4.16 ^a
Glu	26.36 ± 7.04 ^a	38.39 ± 2.61 ^b	24.33 ± 4.53 ^a	36.41 ± 0.46 ^b	32.81 ± 5.97 ^{ab}
Gly	5.09 ± 2.05 ^a	8.04 ± 0.60 ^b	5.11 ± 0.74 ^a	7.67 ± 0.88 ^b	7.93 ± 1.35 ^b
His and Gln	18.74 ± 5.79 ^a	14.96 ± 1.94 ^a	14.79 ± 2.16 ^a	17.15 ± 0.87 ^a	15.05 ± 2.57 ^a
NH ₃	1.18 ± 0.36 ^a	7.44 ± 0.65 ^c	1.19 ± 0.25 ^a	6.50 ± 0.07 ^{bc}	6.29 ± 0.89 ^b
Arg	24.99 ± 7.04 ^b	6.28 ± 0.40 ^a	22.02 ± 3.93 ^b	6.32 ± 0.48 ^a	5.90 ± 1.45 ^a
Thr	14.24 ± 4.71 ^a	17.18 ± 0.72 ^a	12.38 ± 2.38 ^a	17.30 ± 0.90 ^a	15.50 ± 2.73 ^a
Ala	14.44 ± 4.05 ^a	19.64 ± 0.69 ^b	13.91 ± 2.64 ^a	19.73 ± 0.33 ^b	17.40 ± 2.84 ^{ab}
Pro	3.28 ± 1.11 ^a	3.70 ± 0.45 ^a	3.18 ± 0.54 ^a	3.67 ± 0.16 ^a	3.63 ± 0.73 ^a
Cys	2.81 ± 0.69 ^a	2.73 ± 0.13 ^a	2.39 ± 0.46 ^a	2.65 ± 0.22 ^a	2.47 ± 0.47 ^a
Tyr	11.57 ± 2.60 ^a	9.68 ± 0.60 ^a	8.73 ± 1.96 ^a	9.37 ± 0.45 ^a	8.59 ± 1.24 ^a
Val	18.49 ± 4.86 ^a	20.41 ± 1.47 ^a	16.92 ± 3.74 ^a	19.98 ± 0.64 ^a	18.21 ± 2.71 ^a
Met	10.21 ± 2.35 ^a	11.45 ± 0.84 ^a	9.42 ± 2.04 ^a	11.14 ± 0.25 ^a	10.41 ± 1.37 ^a
Lys	29.17 ± 8.68 ^a	34.81 ± 2.25 ^a	27.93 ± 6.79 ^a	33.80 ± 0.73 ^a	30.22 ± 5.64 ^a
Ile	16.05 ± 4.42 ^a	18.36 ± 1.18 ^a	14.94 ± 2.66 ^a	18.12 ± 0.20 ^a	17.07 ± 2.49 ^a
Leu	31.85 ± 8.59 ^a	35.54 ± 2.01 ^a	30.22 ± 6.60 ^a	34.63 ± 0.41 ^a	32.76 ± 4.73 ^a
Phe	15.63 ± 3.71 ^a	16.48 ± 0.88 ^a	14.15 ± 3.20 ^a	16.21 ± 0.21 ^a	15.24 ± 2.09 ^a
Trp	3.64 ± 0.83 ^a	4.18 ± 0.33 ^a	3.17 ± 0.87 ^a	4.14 ± 0.40 ^a	3.95 ± 0.57 ^a
Total	286.11 ± 73.41^a	313.17 ± 19.33^a	259.30 ± 50.69^a	307.44 ± 9.33^a	282.34 ± 46.33^a

^{a,b,c} Different letters in the same row indicate significant differences at $P \leq 0.05$. Values are mean ± standard deviation of three independent replicates (n = 3).

Table 8: Free amino acid contents in control and fermented pork trimmings hydrolysate (mg/10 mL)

Effect of co-inoculation and sequential inoculation of L. fermentum and P. kluyveri on formation of volatile compounds

As seen in Figures 14 (a) to (i), the volatile compound profiles for ethanol, acetic acid, hexanal, 1-hexanol, isoamyl acetate, ethyl acetate, hexyl acetate, 2-phenylethyl acetate, phenylethyl alcohol of pork hydrolysate fermented by *L. fermentum*, *P. kluyveri*, co-inoculation and sequential inoculation were completely different from the unfermented control samples.

Esters were the most abundant compounds in *P. kluyveri*, co- and sequential fermented samples, and alcohols were the major compounds in *L. fermentum* fermented samples.

Hexanal was the dominant volatile compound in the unfermented pork hydrolysate, followed by 2-pentylfuran and 1-pentanol. These dominant compounds imparted an overall undesirable grassy, herbaceous and beany flavours to the control samples.

A large amount of alcohols and acids volatile compounds were identified in samples fermented by *L. fermentum*. As shown in Figures 14 (a) and (b), in *L. fermentum* fermentation, pyruvate was formed from glycolysis and subsequently converted into acetyl-CoA by the pyruvate dehydrogenase complex to produce acetate and ethanol, so the concentration of ethanol and acetic acid were found to have increased gradually during the fermentation. Besides, 1-hexanol (Figure 14 (d)) and hexanoic acid were found, which was mostly derived from enzymatic reduction and oxidation of hexanal in the pork hydrolysate. In all, these identified volatile compounds in *L. fermentum* fermented pork hydrolysate gives it a fresh sour odour.

In summary, an antagonistic relationship was observed between these two cultures, as *P. kluyveri* only proliferated for one day after inoculation and dropped to original levels in the presence of *L. fermentum*, while the latter was stimulated in both inoculation methods compared to their respective monocultures. There were no significant differences ($P < 0.05$) in the final contents of glucose, organic acids, and amino acids between these two inoculation methods. However, inoculation sequence had impacts on the volatile compound formation as more esters were detected in the sequential inoculation.

Example 5 – Effect of pH, xylose addition and temperature on colour and flavour compounds of heat-treated pork trimmings hydrolysate

The heat treatment process of meat sauce is schematically shown in Figure 4.

- 5 The effects of pH, xylose and temperature on the development of colour and aroma compound profile in heat-treated pork trimmings hydrolysate were investigated. Different amounts (0, 0.5, 1.5, 2.5, 3.5 g/100 mL) of xylose were added to the enzymatically hydrolysed pork hydrolysate with pH adjusted to 5.5 or 4.5 prior to heat treatment at 90, 95 or 100 °C for 60 minutes.

10 *Colour*

- The colour changes of controls and heated samples are shown in Tables 9 and 10. All heated samples showed an increase of ΔE regardless of temperatures and pH. The L^* values (lightness) and the a^* values (negative for greenness and positive for redness) were elevated as the sugar content increased. The b^* values (blueness at negative and
15 yellowness at positive) increased at lower sugar contents but either stabilized or decreased at higher sugar contents depending on temperature. At pH 5.5, the three sugar-free samples A90-0, A95-0 and A100-0 had the lowest ΔE values of 2.26, 1.34 and 2.98, respectively. These three sugar-free samples maintained a yellowish transparent colour, which showed no visible colour changes compared to unheated
20 controls. Sample A100-35 showed the highest ΔE value of 23.50 with a transparent dark-brown colour, but there was no significant colour difference compared to sample A90-35 and A95-35 even different ΔE were observed for each sample. Similar trends were found in the Group B samples (pH of 4.5), where the highest ΔE of 25.05 was found in sample B100-35 with a transparent dark-brown colour.

Sample Name #	pH	Sugar added (g/100 mL)	Temperature (°C)	Time (min)
A0	5.5	0	0	0
A90-0	5.5	0	90	60
A90-5	5.5	0.5	90	60
A90-15	5.5	1.5	90	60
A90-25	5.5	2.5	90	60
A90-35	5.5	3.5	90	60
A95-0	5.5	0	95	60
A95-5	5.5	0.5	95	60
A95-15	5.5	1.5	95	60
A95-25	5.5	2.5	95	60
A95-35	5.5	3.5	95	60
A100-0	5.5	0	100	60
A100-5	5.5	0.5	100	60
A100-15	5.5	1.5	100	60
A100-25	5.5	2.5	100	60
A100-35	5.5	3.5	100	60
B0	4.5	0	0	0
B90-0	4.5	0	90	60
B90-5	4.5	0.5	90	60
B90-15	4.5	1.5	90	60
B90-25	4.5	2.5	90	60
B90-35	4.5	3.5	90	60
B95-0	4.5	0	95	60
B95-5	4.5	0.5	95	60
B95-15	4.5	1.5	95	60
B95-25	4.5	2.5	95	60
B95-35	4.5	3.5	95	60
B100-0	4.5	0	100	60
B100-5	4.5	0.5	100	60
B100-15	4.5	1.5	100	60
B100-25	4.5	2.5	100	60
B100-35	4.5	3.5	100	60

There were three replications of each sample treatment for data acquisitions (n = 3).

Table 9: Heat treatment conditions for pork hydrolysate samples

Sample Name #	Colour			
	L^*	a^*	b^*	ΔE
A0	25.11 ± 0.42	-1.54 ± 0.04	1.98 ± 0.29	-
A90-0	27.16 ± 1.42	-1.36 ± 0.03	2.91 ± 0.24	2.26 ± 1.00 A
A90-5	22.82 ± 1.93	-1.24 ± 0.11	6.28 ± 0.27	4.88 ± 1.52 B
A90-15	22.51 ± 1.29	1.74 ± 0.06	14.54 ± 1.69	13.24 ± 1.65 D
A90-25	18.30 ± 3.28	3.42 ± 0.77	18.50 ± 1.32	18.55 ± 3.15 E
A90-35	15.58 ± 2.53	6.16 ± 0.83	19.74 ± 1.80	21.58 ± 2.74 FG
A95-0	24.76 ± 1.11	-1.30 ± 0.06	3.25 ± 0.12	1.34 ± 0.72 A
A95-5	19.28 ± 0.60	-0.56 ± 0.02	9.57 ± 0.08	9.62 ± 0.28 C
A95-15	16.43 ± 2.64	3.85 ± 0.59	17.59 ± 0.94	18.66 ± 2.40 E
A95-25	12.33 ± 2.15	7.76 ± 0.10	16.41 ± 1.03	21.40 ± 1.89 F
A95-35	9.71 ± 2.19	9.77 ± 0.63	13.68 ± 2.23	22.40 ± 2.71 EFG
A100-0	27.52 ± 0.89	-1.60 ± 0.05	3.74 ± 0.55	2.98 ± 0.54 A
A100-5	19.66 ± 1.61	0.72 ± 0.14	13.13 ± 1.07	12.61 ± 1.43 D
A100-15	11.01 ± 0.19	7.35 ± 0.20	15.84 ± 0.33	21.68 ± 0.34 EF
A100-25	6.51 ± 0.30	9.87 ± 0.44	9.59 ± 0.55	23.11 ± 0.57 GH
A100-35	5.17 ± 0.09	9.68 ± 0.28	7.32 ± 0.28	23.50 ± 0.46 H
B0	27.34 ± 0.67	-1.46 ± 0.04	2.97 ± 0.03	-
B90-0	25.02 ± 2.69	-1.24 ± 0.06	2.61 ± 0.40	2.36 ± 2.06 a
B90-5	23.84 ± 1.91	-1.05 ± 0.07	7.32 ± 0.31	5.59 ± 1.28 b
B90-15	19.60 ± 0.84	-0.06 ± 0.20	11.08 ± 0.98	11.29 ± 0.99 d
B90-25	16.70 ± 0.78	1.99 ± 0.30	17.64 ± 0.11	18.45 ± 0.36 ef
B90-35	15.50 ± 0.45	3.72 ± 0.28	17.70 ± 1.74	19.60 ± 1.75 f
B95-0	21.78 ± 0.70	-1.15 ± 0.04	3.12 ± 0.09	5.57 ± 0.10 a
B95-5	20.59 ± 1.08	-0.93 ± 0.03	8.40 ± 0.62	8.68 ± 0.72 c
B95-15	16.68 ± 0.75	1.78 ± 0.28	16.69 ± 0.36	17.67 ± 0.47 e
B95-25	13.44 ± 1.29	5.22 ± 0.17	18.01 ± 1.16	21.55 ± 1.30 g
B95-35	10.57 ± 0.34	7.62 ± 0.14	15.53 ± 0.54	22.84 ± 0.63 g
B100-0	24.05 ± 2.21	-1.36 ± 0.12	3.05 ± 0.82	3.30 ± 1.74 a
B100-5	19.97 ± 1.90	-0.02 ± 0.06	11.96 ± 1.32	11.71 ± 1.78 d
B100-15	12.50 ± 0.79	6.08 ± 0.22	17.78 ± 1.12	22.28 ± 1.12 g
B100-25	9.15 ± 0.54	9.18 ± 0.13	13.58 ± 0.57	23.59 ± 0.58 gh
B100-35	6.55 ± 0.51	10.74 ± 0.70	9.80 ± 0.89	25.05 ± 1.14 h

All values are mean ± standard deviation with three replications of each sample treatment ($n = 3$). Different letters (capital letters or lowercase letters) within the same column indicate significant difference between/among each other ($P < 0.05$).

- 5 "Before pH" refers to the adjusted pH of samples before heat treatment.

Table 10: Colour changes of control and heat-treated pork hydrolysates

pH

As shown in Figure 15, the pH of samples decreased during heat treatment. The lowest pH in each group was found to be 4.86 and 4.35 in samples A100-35 and B100-35, respectively. The lowered pH during the Maillard reaction reflects the depletion of the amino group and the formation of organic acids. In many studies, the lowered pH is also an indicator of the browning process.

Reducing sugar/xylose content

Before heat treatment, the total sugar content in the original pork hydrolysate was at a trace level, so reducing sugar (xylose) was added to boost the Maillard reaction. As shown in Figure 16, xylose reduction generally occurred during heat treatment, regardless of temperature, pH and the amount of sugar. At pH 5.5, the least sugar reduction was found in sample A90-15 at 2.18 mg/mL, while the most sugar reduction was found in sample A100-35 at 5.75 mg/mL (Figure 16 (a)). At pH 4.5, the numbers were 2.48 mg/mL for sample B90-35 and 5.85 mg/mL for sample B100-35 (Figure 16 (b)). These results showed little differences in sugar consumption among samples treated under different conditions.

Free amino acids

Free amino acids of the hydrolysate played an important role in the formation of flavour and pigmented compounds in heat-treated samples. An objective of the present invention is to develop a novel meat sauce with savoury flavour that reflects the meaty and sweet aroma of well-cooked meat. As seen in Figure 17, sulfur-containing amino acids such as cysteine (Cys), and other important precursors such as proline (Pro), methionine (Met), phenylalanine (Phe), which are associated with the formation of cooked potato, vegetable-like, roasted and honey-like sweet odours, are present.

As shown in Figures 17 (i) and (j), the total amount of amino acids showed no significant changes after heat treatment for both pH 4.5 and pH 5.5 samples, maintaining at approximately 300 mg/10 mL.

Volatile compounds

To understand the flavour compound formation in the Maillard reaction of pork hydrolysate, the volatile compounds found in controls and heat-treated samples are summarized. The volatile compounds were divided into five major groups: acids, alcohols, aldehydes, ketones, and furans. Three identified dominant compounds were
5 hexanal, furfural and 2-pentylfuran (Figure 18).

Hexanal accounted for 29 % and 28 % RPA for controls of pH 5.5 and pH 4.5, and it was significantly decreased after heat treatment (Figures 18 (a) and (b)). The lowest peak area of hexanal detected was 9.44×10^6 in sample A100-35 and 7.94×10^6 in sample B100-35, accounting for 1.9 % and 1.2 % RPA of each sample. Hexanal is an
10 aliphatic aldehyde that gives off a rancid and green-grassy odour of the untreated pork hydrolysate.

Contrary to hexanal, the amount of furfural increased significantly after the heat treatment (Figure 18 (c) and (d)). The peak areas of furfural were only 4.13×10^6 and 9.14×10^6 for pH 5.5 and pH 4.5 controls, accounting for 2.4 % and 3.5 % RPA,
15 respectively. In heat-treated samples, the highest peak areas were found in A100-35 (111.67×10^6) and B100-35 (361.74×10^6), accounting for 38.8 % and 55.7 % RPA, which respectively increased by around 27 and 40 folds compared to the controls. Furfural is an intermediate (no sulfur) produced in the Maillard reaction with the presence of a pentose, and gives a strong baked-potato, spicy, and burnt note. The
20 formation of furfural is either from the enolization of Amadori compounds or the dehydration of sugar, and both these reactions could be enhanced at acidic and high-temperature conditions. During meat cooking, furfural often reacts with the hydrogen sulphide, which is formed from the cysteine breakdown, to produce 2-furanmethanethiol that gives a strong and distant "roasted meat" aroma to the product
25 (Xu et al., 2011). However, 2-furanmethanethiol was undetected in this study, which might be due to the low concentration of Cys available in the pork hydrolysate.

The level of 2-pentylfuran significantly increased in heat-treated samples compared to controls, with the highest peak area found in sample A100-5 at 272.50×10^6 , accounting for 57.1 % RPA (Figure 18 (e) and (f)). Interestingly, the formation of 2-
30 pentylfuran in heat treatment had no correlation to the sugar content and temperature. 2-Pentylfuran is usually formed from the oxidation of linoleic acid and gives fruity, floral, butter, green and beany notes to the foods. Although 2-pentylfuran produced in the

heat treatment did not directly give a desirable “meat-like” odour, it has been recognized as a contributor to the overall odour of broiled or roasted-meat to some sauce products.

In summary, heat treatment eliminated the dominant off-flavour compound, hexanal, in
5 pork hydrolysate, and a higher temperature and xylose content promoted the generation of furfural and 2-pentylfuran, which possess a roasted and sweet flavour note. More furfural was detected in at pH 4.5 during heat treatment. However, the desirable savoury “meat-like” sulfur-containing and nitrogen-containing aroma compounds were not detected, which might be due to deficient levels of
10 cysteine/cystine in the pork hydrolysate.

Whilst the foregoing description has described exemplary embodiments, it will be understood by those skilled in the technology concerned that many variations may be made without departing from the present invention.

References

- 15 A.O.A.C. (1995). Official method of analysis. 16th Edition, Association of Official Analytical Chemists, Washington DC. Retrieved from: https://www.aoac.org/AOAC_Prod_Imis/AOAC_Member/Default.aspx (Accessed 22 Jan 2019);
- 20 Bartkieni , E., et al, *Frontiers in Microbiology*, 2019, 1036: 1-4;
- Dey, S. S., & Dora, K. C., *Journal of Food science and Technology*, 2014, 51(1): 16-24;
- Gao, P., et al, *International Journal of Food Science & Technology*, 2016, 51(7): 1630-
25 1637;
- Kwan, T. H., et al, *Bioresource Technology*, 2016, 217: 129-136;
- Lape a, D., et al, *Journal of Agricultural and Food Chemistry*, 2018, 66(37): 9738-
30 9749;
- Law, M. R., *American Journal of Hypertension*, 1997, 10(S4): 42S-45S;
- Lee, P. R., et al, *Nutrition & Food Science*, 2013, 43(3): 267-276;
- 35 Meeker, D. L., *Revista Brasileira de Zootecnia*, 2009, 38: 432-440;
- Nguyen, H. T. M., et al, *Food Technology and Biotechnology*, 2011, 49(1): 48;
- 40 Oh, M., et al, *Meat Science*, 2016, 119: 16-21;

Ohata, M., et al, *Food Chemistry*, 2016, 194: 1034-1039;

Reig, M., Aristoy, M. C., & Toldrá, F., *Food Chemistry*, 2013, 140(3): 478-482;

5

Trang, N. H., et al, *Journal of the Science of Food and Agriculture*, 2005, 85(11): 1829-1837;

Nilsang, S., et al, *Journal of Food Engineering*, 2005, 70(4): 571-578;

10

Toh, M., & Liu, S. Q., *Journal of Applied Microbiology*, 2017, 123(4): 956-968;

Vong, W. C., & Liu, S. Q., *Journal of the Science of Food and Agriculture*, 2017, 97(1): 135-143;

15

Wood, I. P., Elliston, et al, *Biomass and Bioenergy*, 2012, 44: 117-121;

Yoshikawa, S., et al, & Ohta, T., *Journal of Agricultural and Food Chemistry*, 2010, 58(10): 6410-6417.

20