

An okara-based beverageTechnical Field

The present invention relates to an okara-based beverage. In particular, the okara-based beverage may comprise probiotics.

Background

Okara is a food processing by-product from soymilk and tofu manufacture. Globally, soy food companies produce up to millions of tons of okara annually, and typically the okara is disposed. While okara-soy products have been formulated, they have not been popular with amongst consumers due to the undesirable texture and flavour of okara.

Summary of the invention

The present invention seeks to address these problems, and/or to provide an improved okara-based beverage, as well as an improved method for biotransforming okara into a suitable beverage without generating any waste stream.

In general terms, the invention relates to an okara-based beverage comprising probiotics, which may have health benefits on the consumer. Further, the okara-based beverage of the present invention may be more palatable due to the biotransformation of okara by way of making the beverage according to the method of the present invention in order to reduce the amount of insoluble dietary fibre (IDF) and increase the amount of esters in the beverage.

According to a first aspect, the present invention provides an okara-based probiotic beverage comprising probiotics, soluble dietary fibre, free isoflavones and esters, wherein the probiotics have a cell count of $\geq 5.0 \log \text{ CFU/mL}$. In particular, the probiotics may have a cell count of $\geq 6.0 \log \text{ CFU/mL}$.

The probiotics may be any suitable probiotics for the purposes of the present invention. According to a particular aspect, the probiotics may comprise lactobacilli, bifidobacteria, *Saccharomyces* yeast, or a combination thereof. In particular, the probiotics may comprise lactobacilli selected from, but not limited to, *Lactobacillus* (*Lb.*) *acidophilus*, *Lb. casei*, *Lb. paracasei*, *Lb. rhamnosus*, *Lb. helveticus*, *Lb. plantarum*, or a combination thereof. In particular, the probiotics may comprise *Saccharomyces*

boulardii, *Saccharomyces cerevisiae*. Even more in particular, the probiotics may comprise *Saccharomyces cerevisiae* CNCM I-3856.

According to a particular aspect, after 6 weeks of storage, the probiotics comprised in the beverage has a cell count of ≥ 5 log CFU/mL and retains at least: 70% of the
5 soluble dietary fibre; 90% of the free isoflavones; and 20% of the esters.

The beverage may comprise a suitable amount of esters. For example, the beverage may comprise 0.1 – 100 μg esters/g dried okara (equivalent to 5 – 5000 $\mu\text{g/L}$ in a beverage containing 5% w/w dried okara).

The beverage may comprise a suitable amount of free isoflavones. For example, the
10 beverage may comprise 50 – 500 μg free isoflavones/g dried okara (equivalent to 2.5 – 25 mg/L in a beverage containing 5% w/w dried okara).

The beverage may further comprise additional additives. The additive may be any suitable additive. For example, the additive may be, but not limited to, sweetener, flavouring, stabilizer, colourant, preservatives, acidity regulator, or a combination
15 thereof.

According to a second aspect of the present invention, there is provided a method of forming an okara-based probiotic beverage described above, the method comprising:

- treating okara with carbohydrase to form treated okara;
- adding probiotics and a yeast to the treated okara; and
- 20 - fermenting the treated okara for a pre-determined period of time at a pre-determined temperature to form the okara-based probiotic beverage.

In particular, the method of the present invention is a zero-waste method.

25 According to a particular aspect, the treating may comprise carbohydrase hydrolysis. The carbohydrase used in the treating may be any suitable carbohydrase for the purposes of the present invention. For example, the carbohydrase may be, but not limited to, cellulase, hemicellulase, pectinase, pentosanase, arabanase, xylanase, mannase, glycosidase, or a combination thereof.

The probiotics used in the method may be any suitable probiotics. For example, the probiotics may be as described above in respect of the first aspect of the present invention.

5 The yeast used in the method may be any suitable yeast. For example, the yeast may be *Saccharomyces* yeast, non-*Saccharomyces* yeast, or a combination thereof. In particular, the yeast may be, but not limited to, *Saccharomyces* (*S.*) *cerevisiae*, *Torulaspora* (*T.*) *delbrueckii*, *Pichia* (*P.*) *kluyveri*, *Lindnera* (*L.*) *saturnus*, or a combination thereof.

10 The adding may comprise adding a suitable amount of probiotics and a suitable amount of yeast. According to a particular aspect, the adding may comprise adding probiotics to obtain an initial probiotic count of 5-7 log CFU probiotics/mL. According to a particular aspect, the adding may comprise adding yeast to obtain an initial yeast count of 5 log CFU yeast/mL.

15 The fermenting may be carried out under suitable conditions. According to a particular aspect, the pre-determined period of time may be 8-96 hours. According to a particular aspect, the pre-determined temperature may be 15-45°C.

According to a particular aspect, the method may further comprise adjusting pH of the okara prior to the treating.

20 According to a particular aspect, the method may further comprise heat-treating the okara prior to the treating. The heat-treating may be to a suitable temperature.

According to a particular aspect, the method may further comprise cooling the treated okara to the pre-determined temperature prior to the adding.

Brief Description of the Drawings

25 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 changes in insoluble and soluble fibre in different treatments - E = enzyme-treated okara; EL = enzyme-treated okara fermented with probiotics *Lb. casei*;

EY = enzyme-treated okara fermented with yeast *L. saturnus*. Different letters indicate significant differences in insoluble fibre (lower case letters) and soluble fibre (upper case letters) at $p < 0.05$;

Figure 2 shows the changes in glycosidic and free isoflavones in different treatments (n = 3) where E = enzyme-treated okara; EL = enzyme-treated okara fermented with probiotics *Lb. casei*; EY = enzyme-treated okara fermented with yeast *L. saturnus*; EYL = enzyme-treated okara fermented with probiotics *Lb. casei* and yeast *L. saturnus*. Different letters indicate significant differences in glycosidic isoflavones (lower case letters) and free isoflavones (upper case letters) at $p < 0.05$;

Figure 3 shows the changes in total aldehydes and total esters in different treatments (n = 3) where E = enzyme-treated okara; EL = enzyme-treated okara fermented with probiotics *Lb. casei*; EY = enzyme-treated okara fermented with yeast *L. saturnus*; EYL = enzyme-treated okara fermented with probiotics *Lb. casei* and yeast *L. saturnus*. ND = not detected. Different letters indicate significant differences in total aldehydes (lower case letters) and total esters (upper case letters) at $p < 0.05$;

Figure 4 shows the effect of heating on soluble fibre in okara and carbohydrase-treated treatments. Different letters (a, b, c) indicate a significant difference between treatments; and

Figure 5 shows the effect of carbohydrase and heating on isoflavone glycosides (unfilled bars) and aglycones (filled bars). The different letters (a, b, c, A, B, C) indicate a significant difference between the isoflavone glycosides or aglycones between the treatments.

Detailed Description

As explained above, there is a need for processing okara to better utilise okara. At present, okara is used for composting, as animal feed or dumped directly into landfills. However, okara retains many nutrients. On a dry basis, okara contains 40-50% w/w insoluble dietary fibres (IDF), 4-15% w/w soluble dietary fibres (SDF) and 15-30% w/w protein. Accordingly, the present invention relates to an okara-based beverage which fully utilises okara and achieves practically zero waste.

In general terms, the present invention relates to an okara-based beverage and a method of forming the same. The okara-based beverage may have health benefits as the beverage has high cell counts of live probiotics, soluble fibre, significantly less insoluble fibre, and greater amounts of free amino acids, free isoflavones (isoflavone
5 aglycones) and fruity esters. Further, after 6-8 weeks of storage, the probiotic viability in the beverage did not significantly change when stored at low temperature and decreased minimally when stored at ambient temperature. In fact, the probiotic viability exceeded the minimum dosage for conferring health benefits.

According to a first aspect, the present invention provides an okara-based probiotic
10 beverage comprising probiotics, soluble dietary fibre, free isoflavones and esters, wherein the probiotics have a cell count of ≥ 5.0 log CFU/mL.

The probiotics comprised in the beverage may be any suitable probiotics. The probiotics may be any suitable live microorganism which, when provided in an adequate amount, confers a health benefit on the host. For example, the probiotic
15 bacteria may comprise lactobacilli, bifidobacteria, *Saccharomyces* yeast, or a combination thereof. In particular, the probiotics may comprise lactobacilli selected from, but not limited to, *Lactobacillus (Lb.) acidophilus*, *Lb. casei*, *Lb. paracasei*, *Lb. rhamnosus*, *Lb. helveticus*, *Lb. plantarum*, or a combination thereof. Even more in particular, the lactobacilli may comprise *Lb. plantarum* 299v, *Lb. helveticus* L10, *Lb.*
20 *paracasei* L26, *Lb. rhamnosus* LGG, or a combination thereof. In particular, the probiotics may comprise *Saccharomyces boulardii*, *Saccharomyces cerevisiae*. Even more in particular, the probiotics may comprise *Saccharomyces cerevisiae* CNCM I-3856.

A suitable amount of probiotics may be comprised in the okara-based beverage. For
25 example, the probiotics may have a cell count of ≥ 5.0 log CFU/mL. According to a particular aspect, the probiotics may have a cell count of ≥ 6.0 log CFU/mL. Even more in particular, the probiotics may have a cell count of ≥ 7.0 log CFU/mL.

In particular, the probiotics comprised in the beverage may have a cell count of 5.0-10.0 log CFU/mL, 5.5-9.5 log CFU/mL, 6.0-9.0 log CFU/mL, 6.5-8.5 log CFU/mL, 7.0-
30 8.0 log CFU/mL. Even more in particular, the probiotics comprised in the beverage may have a cell count of about 7.0-10.0 log CFU/mL.

The beverage may comprise a suitable amount of esters. For example, the beverage may comprise 0.1 – 100 µg esters/g dried okara (equivalent to 5 – 5000 µg/L in a beverage containing 5% w/w dried okara). The presence of esters in the beverage show that the beverage has undergone fermentation. In particular, the ester comprised
5 in the beverage may impart a fruity and/or floral characteristic to the beverage. This may be advantageous as the esters may help to mask the beany and/or grassy odour which may have been originally present in the okara.

The esters comprised in the beverage may be any suitable ester. For example, the ester may be, but not limited to, ethyl esters, acetate esters, or a combination thereof.
10 In particular, the esters may be, but not limited to ethyl acetate, ethyl hexanoate, ethyl heptanoate, 2-phenylmethyl acetate, 2-phenylethyl acetate, isobutyl acetate, 1-butyl-3-methyl-acetate (isoamyl acetate), hexyl acetate, hexyl hexanoate, heptyl acetate, or a combination thereof.

The beverage may comprise a suitable amount of free isoflavones. The free
15 isoflavones may be referred to as isoflavone aglycone. For example, the beverage may comprise 50 – 500 µg free isoflavones/g dried okara (equivalent to 2.5 – 25 mg/L in a beverage containing 5% w/w dried okara).

For the purposes of the present invention, free isoflavones is defined as a plant-based bioactive phytoestrogenic compound without a sugar molecule attached to it. The free
20 isoflavones may include, but is not limited to, daidzein, glycitein and genistein. The free isoflavones comprised in the beverage are beneficial as they are more readily absorbed in the gastrointestinal tract of the consumer of the beverage. Soy isoflavones are phytoestrogens which have anti-carcinogenic, antioxidant and anti-atherosclerotic activities. Free isoflavones are suggested to be more bioavailable in humans than their
25 glycosidic form in fermented soy foods.

According to a particular aspect, the beverage may have a total isoflavone content of 0.2 – 1 mg total isoflavones/g dried okara (equivalent to 10 – 50 mg/L in a beverage containing 5% w/w dried okara). The total isoflavones measures the total amount of isoflavones consisting of free isoflavones and bound isoflavones (isoflavone glycoside)
30 in the beverage.

According to a particular aspect, after 6-8 weeks of storage, the probiotics comprised in the beverage may have a cell count of ≥ 5 log CFU/mL and may retain at least: 70% of the soluble dietary fibre; 90% of the free isoflavones; and 20% of the esters, as compared to the originally produced beverage.

- 5 In particular, when the beverage is stored at ambient temperature for 6-8 weeks, the probiotics comprised in the beverage may have a cell count of ≥ 7 log CFU/mL and may retain almost all the soluble dietary fibre, free isoflavones and esters as the originally produced beverage.

- 10 In particular, when the beverage is stored at a low temperature, such as about 5-7°C, the probiotics comprised in the beverage may have a cell count of ≥ 7 log CFU/mL and may retain at least: 70% of the soluble dietary fibre; 90% of the free isoflavones; and 20% of the esters, as compared to the originally produced beverage.

- 15 Accordingly, it can be seen that the beverage may still confer health benefits to the consumer even after a certain period of time following the manufacture of the beverage. Thus, the beverage may have a suitable shelf-life.

- 20 The beverage may be stored at a suitable temperature so as to maintain the probiotics at a suitable level. For example, the beverage may be stored at a temperature of about $\leq 25^\circ\text{C}$. Preferably, the beverage may be stored at a temperature of $\leq 20^\circ\text{C}$. In particular, the beverage may be stored at a temperature of about 1-20°C, 5-15°C, 7-10°C.

The beverage may further comprise additional additives. The additive may be any suitable additive for giving a more finished consumer product. According to a particular aspect, the additive may be, but not limited to, sweetener, flavouring, stabilizer, thickeners, colourant, preservatives, acidity regulator, or a combination thereof.

- 25 According to a second aspect of the present invention, there is provided a method of forming an okara-based probiotic beverage described above, the method comprising:

- treating okara with carbohydrase to form treated okara;
- adding probiotics and a yeast to the treated okara; and

- fermenting the treated okara for a pre-determined period of time at a pre-determined temperature to form the okara-based probiotic beverage.

5 In particular, the method of the present invention is a zero-waste method. In other words, the method of the present invention does not produce any waste and the okara is completely utilised in the making of the okara-based beverage. Accordingly, the method of the present invention overcomes the problem of finding suitable ways of disposing the okara formed from soymilk and tofu manufacturing, and additionally,
10 forms a useful and beneficial beverage.

The okara used for the purposes of the present invention may be any suitable okara. The okara may undergo treatment before treating the okara with carbohydrase. According to a particular aspect, the method may further comprise mixing the okara with water to form an aqueous slurry of okara. Any suitable amount of okara may be
15 added to form the slurry. For example, the amount of okara may be 1-10 % w/w (dry weight basis of okara). In particular, the amount of okara may be about 2-5 % w/w. Even more in particular, the amount of okara may be about 5 % w/w.

The method may further comprise adjusting the pH of the okara prior to the treating. In particular, the pH may be adjusted to the working pH of the carbohydrase used in the
20 treating. Accordingly, the adjusting the pH enables the carbohydrase to effectively function in treating the okara. For example, the pH may be adjusted to a pH of 2-7. The adjusting the pH may be by any suitable method. For example, the adjusting may comprise adding a suitable acid. The acid used for the adjusting must be an acid which is suitable for being consumed. In particular, the acid may be, but not limited to, malic
25 acid, citric acid, lactic acid, tartaric acid, gluconic acid, succinic acid, or a combination thereof.

According to a particular aspect, the method may further comprise heating the okara prior to the treating. The heating may comprise mild pasteurization of the okara. The heating may extend the shelf life of the okara prior to the treating and may also reduce
30 the risk of contamination during the treating. The heating may be carried out under suitable conditions. For example, the heating may be carried out at a temperature of about 60-140°C. In particular, the temperature may be about 100-125°C.

The heating may be carried out for a suitable period of time. For example, the heating may be for 2 seconds-60 minutes. In particular, the heating may be for about 10-30 minutes. Even more in particular, the heating may be for about 15-20 minutes.

According to a particular aspect, the okara slurry may be cooled prior to the treating.

5 The carbohydrase used in the treating may be any suitable carbohydrase for the purposes of the present invention. For example, the carbohydrase may be, but not limited to, cellulase, hemicellulase, pectinase, pentosanase, arabanase, xylanase, mannase, glycosidase, or a combination thereof. A suitable amount of carbohydrase may be added to the okara slurry.

10 The treating may comprise carbohydrase hydrolysis. The carbohydrase hydrolysis may be under suitable conditions. For example, the carbohydrase hydrolysis may be for a suitable period of time and at a suitable temperature. In particular, the carbohydrase hydrolysis may be for 2-12 hours, 3-10 hours, 4-9 hours, 5-8 hours, 6-7 hours. Even more in particular, the hydrolysis may be for about 3 hours. The hydrolysis may be
15 carried out at a temperature of about 30-70°C, 35-65°C, 40-60°C, 45-55°C, 48-50°C. Even more in particular, the hydrolysis may be carried out at a temperature of about 50°C.

The treating reduces the amount of IDF in the okara significantly and increases the amount of SDF. In particular, the carbohydrase breaks down the okara IDF into SDF
20 (oligosaccharides) and simple sugars, leading to an increase in SDF, glucose and galactose. Further, the SDF may be a good source of prebiotic to support probiotic growth during the subsequent fermenting step.

Following the treating, the treated okara may be formed. The treated okara may be heated for enzyme denaturation and sterilisation. The heating may be carried out under
25 suitable conditions. For example, the heating may be carried out at a suitable temperature. The temperature may be dependent on the carbohydrase used in the treating. In particular, the temperature may be about 90-140°C. Even more in particular, the temperature may be about 100-125°C.

The heating may be carried out for a suitable period of time. For example, the heating
30 may be for 1-30 minutes. In particular, the heating may be for about 15-20 minutes.

The method may further comprise cooling the heated treated okara prior to the adding of the probiotics and yeast.

The adding may comprise adding suitable probiotics. For example, the probiotics may be lactobacilli, bifidobacteria, *Saccharomyces* yeast, or a combination thereof. In particular, the probiotics may comprise lactobacilli selected from, but not limited to, 5 *Lactobacillus (Lb.) acidophilus*, *Lb. casei*, *Lb. paracasei*, *Lb. rhamnosus*, *Lb. helveticus*, *Lb. plantarum*, or a combination thereof. Even more in particular, the lactobacilli may comprise *Lb. plantarum* 299v, *Lb. helveticus* L10, *Lb. paracasei* L26, *Lb. rhamnosus* LGG, or a combination thereof. In particular, the probiotics may comprise 10 *Saccharomyces boulardii*, *Saccharomyces cerevisiae*. Even more in particular, the probiotics may comprise *Saccharomyces cerevisiae* CNCM I-3856.

The adding may comprise adding a suitable amount of probiotics. Probiotics may be added so that the initial probiotic count in the treated okara may be ≥ 5.0 log CFU/mL. For example, the amount of probiotics added may be 5-9 log CFU/mL. In particular, the 15 amount of probiotics added may be about 6-8 log CFU/mL, 6.5-7 log CFU/mL. Even more in particular, the amount of probiotics added may be 5-7 log CFU/mL. According to a particular aspect, the adding may comprise adding probiotics to obtain an initial probiotic count of 7 log CFU probiotics/mL.

The adding may comprise adding a suitable yeast. For example, the yeast may be 20 *Saccharomyces* yeast, non-*Saccharomyces* yeast, or a combination thereof. In particular, the yeast may be, but not limited to, *Saccharomyces (S.) cerevisiae*, *Torulaspora (T.) delbrueckii*, *Pichia (P.) kluyveri*, *Lindnera (L.) saturnus*, or a combination thereof.

The adding may comprise adding a suitable amount of yeast. For example, the amount 25 of yeast added may be 3-9 log CFU/mL. In particular, the amount may be about 5-7 log CFU/mL. According to a particular aspect, the adding may comprise adding yeast to obtain an initial yeast count of 5 log CFU yeast/mL.

The adding probiotics and the adding a yeast may be carried out simultaneously or sequentially.

The combination of the probiotics and the yeast may be particularly selected. The selection of probiotics and yeast may be based on the ability of the yeast to: generate pleasant-smelling esters during fermentation; and support probiotic viability during ambient and chilled temperature storage.

- 5 The fermenting may be carried out under suitable conditions. For example, the fermenting may be at a pre-determined temperature for a pre-determined period of time. The pre-determined period of time may be any suitable period of time for the purposes of the present invention. According to a particular aspect, the pre-determined period of time may be 8-96 hours. In particular, the pre-determined period of time may be about 10-90 hours, 12-84 hours, 18-72 hours, 24-60 hours, 36-48 hours. Even more
10 in particular, the pre-determined period of time may be about 24-48 hours.

The pre-determined temperature may be any suitable temperature for the purposes of the present invention. According to a particular aspect, the pre-determined temperature may be 15-45°C. In particular, the pre-determined temperature may be 20-40°C, 25-
15 35°C, 30-34°C. Even more in particular, the pre-determined temperature may be about 30°C. The temperature may be changed at any point during the fermenting.

According to a particular aspect, the formed okara-based probiotic beverage may be stored at a suitable temperature following the fermentation. For example, the beverage may be stored at a temperature of $\leq 30^{\circ}\text{C}$. In particular, the beverage may be stored at
20 a temperature of about $\leq 25^{\circ}\text{C}$. Even more in particular, the beverage may be stored at a temperature of about $1-5^{\circ}\text{C}$.

The method of the present invention has several advantages. For example, the treating with carbohydrase converts the insoluble fibres in okara into soluble fibres. The latter also act as prebiotics to support the growth of probiotics subsequently. The treated
25 okara acts as a rich fermentation media, rather than a simple delivery vehicle or carrier for probiotics. The reduction of insoluble fibre in the okara slurry also makes it flow more easily, which is nutritionally and physically more desirable in a beverage.

During the fermenting, the yeast converts the unpleasant off-odourants aldehydes naturally present in okara into esters, changing the aroma profile of the okara from
30 green and grassy to pleasant and fruity. The yeast also supports probiotics viability

during ambient and chilled storage for at least 6-8 weeks, improving the nutritional value of okara. This interaction is unexpected in a medium containing okara.

Both yeast and probiotics act in synergy to transform the bound isoflavones (less bioavailable) into free isoflavones (more bioavailable).

- 5 Only selected combinations of probiotics and yeast strains will result in the aforementioned positive effects.

According to another aspect, there is provided the okara-based probiotic beverage as described above for use in medicine. In particular, the okara-based probiotic beverage of the present invention may be for use in improving the gut health and/or digestion of a
 10 consumer of the beverage and other health benefits conferred by specific probiotics used which may include, but is not limited to, enhancing immune response, help with antibiotic-associated diarrhoea and reducing mucosal inflammation.

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

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

20 EXAMPLE

Example 1

Material	Source
<i>Lactobacillus casei</i> (<i>Lb. casei</i>) LAFTI L26	Originally from DSM (L26 now owned by Lallemand)
<i>Lindnera saturnus</i> (<i>L. saturnus</i>) NCYC 22	National Collection of Yeast Cultures, UK
Enzymes (carbohydrases Celluclast 1.5L, Viscozyme L, Pectinex Ultra SP-L)	Novozymes, Denmark
Okara	Super Bean International Pte Ltd

Method

To obtain the okara (soybean residue/pulp), soaked soybeans were ground into fine particles before filtering, and the residue was collected. Okara was added to water at 5 % w/w (dry weight basis of okara) to obtain an aqueous slurry, which was then
5 adjusted to the optimum pH of selected carbohydrase. The slurry was heat-treated at 121°C, 15 minutes and cooled. The carbohydrase was then added at 3% v/v enzyme:substrate (dry weight basis of okara), and carbohydrase hydrolysis was carried out at 50°C, 150 rpm for 3 hours.

The treated slurry was then cooled to 30°C before microbial inoculation. Preferably, the
10 carbohydrase used contains at least one of the following: cellulase, hemicellulase and/or pectinase. These enzymes (Celluclast 1.5L, Viscozyme L, Pectinex Ultra SP-L, all from Novozymes) have been shown to effectively break down the okara insoluble fibre.

To the cooled treated okara slurry, probiotics and yeast were added to achieve an
15 initial cell count of about 7 log CFU probiotics/mL and 5 log CFU yeast/mL. Fermentation was then conducted at 30°C, 48 hours. The final product obtained was then further analysed for dietary fibre content, isoflavone content, aroma and its shelf life (based on probiotic viability) at ambient and chilled temperatures.

Results

20 The fermentation with probiotic *Lb. casei* L26 and yeast *L. saturnus* NCYC 22 had resulted in favourable effects. Carbohydrase treatment of the okara slurry reduced its insoluble fibre significantly, such that the beverage had better flow properties and a less gritty mouthfeel. The amount of soluble fibre also increased, which acted as a good source of prebiotic to support probiotic growth during fermentation, as observed
25 from its consumption by the probiotic (see Figure 1).

After fermentation, the beverage contained about 6.5 log CFU yeast/mL and 9 log CFU probiotic/mL. The recommended dose is to consume at least 9 log CFU live probiotics per serving of product in order for health benefits to be exerted in a host. In a 100 mL serving, the okara probiotic beverage contained 11 log CFU probiotics, which greatly
30 exceeded the recommended dose.

Further, the okara beverage also contained considerable amounts of free isoflavones, as shown in Figure 2, as well as a greater amount of esters as shown in Figure 3. The addition of yeast converted the aldehydes into esters.

The yeast also supported probiotic viability during ambient storage. This is shown in

5 Table 1.

Treatment	<i>Lb. casei</i> cell count (log CFU/mL)		
	Week 0	Week 6	
		5°C	25°C
EL	9.20±0.01	9.29±0.05	7.64±0.54
EYL	9.23±0.01	9.27±0.02	7.88±0.45

Key: EL=enzyme-treated okara fermented with probiotic *Lb. casei*
 EYL=enzyme-treated okara fermented with probiotic *Lb. casei* and yeast
L. saturnus

10 **Table 1: Change in probiotic cell count in treatments over 6 weeks storage at different temperatures**

When stored at 5°C for 6 weeks, the amount of probiotics remained at 9.27 log CFU/mL, almost unchanged from its initial count. When stored at 25°C for 6 weeks, the amount of probiotics remained at 7.88 log CFU/mL. This is equivalent to 9.88 log
 15 CFU/100 mL, still above the recommended dose of 9 log CFU per serving. Thus, it can be seen that the okara-based beverage has a long and stable shelf-life based on probiotic viability).

Example 2

Material

Code	Material	Source
	Carbohydrase	
CC	Celluclast® 1.5L	Novozymes, Denmark
PX	Pectinex® Ultra SP-L	Novozymes, Denmark
VZ	Viscozyme® L	Novozymes, Denmark
	Probiotic	
P1	<i>Lb. plantarum</i> 299v	Probi AB, Sweden
P2	<i>Lb. helveticus</i> L10	Lallemand, Canada
P3	<i>Lb. paracasei</i> L26	Lallemand, Canada
P4	<i>Lb. rhamnosus</i> LGG	Chr. Hansen, Denmark
	Yeast	

Y1	<i>Saccharomyces cerevisiae</i> EC-1118	Lallemand, Canada
Y2	<i>Pichia kluyveri</i> FrootZen	Chr. Hansen, Denmark
Y3	<i>Lindnera saturnus</i> NCYC 22	National Collection of Yeast Cultures, UK
Y4	<i>Torulaspora delbrueckii</i> Prelude	Chr. Hansen, Denmark
	Others	
-	Okara	Super Bean International Pte. Ltd.

Combinations

Different combinations of carbohydrase, probiotics and yeast were used for conducting the experiments. The different combinations are as shown in Table 2. In Table 2, reference to carbohydrase-unheat refers to okara treated with carbohydrase and for which analysis was done before denaturation of the carbohydrase. Carbohydrase-heat refers to okara treated with carbohydrase and for which analysis was done after denaturation of carbohydrase. This was the control for all fermented treatments. In all the fermented treatments, the carbohydrase was denatured by heating.

Set up code	Carbohydrase	Probiotic	Yeast
CC (unheated)	CC-unheat		
PX (unheated)	PX-unheat		
VZ (unheated)	VZ-unheat		
CC (heated)	CC-heat	-	-
PX (heated)	PX-heat	-	-
VZ (heated)	VZ-heat	-	-
All_1	CC	P3	Y1
All_2	CC	P2	Y4
All_3	CC	P4	Y2
All_4	PX	P1	Y2
All_5	PX	P2	Y3
All_6	VZ	P3	Y3
All_7	VZ	P1	Y4
All_8	VZ	P4	Y1
Co_2	-	P2	Y4
Co_4	-	P1	Y2
Co_6	-	P3	Y3
Co_8	-	P4	Y1
MonoP_1	CC	P3	-
MonoP_3	CC	P4	-
MonoP_5	PX	P2	-
MonoP_7	VZ	P1	-
MonoY_1	CC	-	Y1
MonoY_3	CC	-	Y2
MonoY_5	PX	-	Y3
MonoY_7	VZ	-	Y4

Table 2: Combinations of carbohydrase, probiotic bacteria and yeast used

Method

To obtain the okara (soybean residue/pulp), soaked soybeans were ground into fine particles before filtering, and the residue was collected. Okara was added to water at 5 % w/w (dry weight basis of okara) to obtain an aqueous slurry, which was then adjusted to the optimum pH of selected carbohydrase. For example, for CC, PX and VZ, the optimum pH was 5, 3.5 and 4.5, respectively.

The carbohydrase was then added at 3% v/v enzyme:substrate (dry weight basis of okara), and carbohydrase hydrolysis was carried out at the carbohydrase optimal

temperature at 150 rpm for 3 hours. The temperature for the carbohydrase hydrolysis was 50°C for CC and VZ, and 35°C for PX.

The treated slurry was then heat treated for enzyme denaturation and sterilisation. In particular, CC and VZ- combinations were heated to 121°C for 15 minutes and PX-combinations were heated at 115°C for 20 minutes.

After cooling, yeast and probiotics were each added at 1% v/v into the slurry and mixed well. The initial probiotic count was about 7 log CFU/mL, while the initial yeast count was about 5 log CFU/mL. Fermentation was then conducted at 30°C for 24 hours. The final product obtained was then further analysed for probiotic viability at both 25°C and 10°C (indicative of its shelf life during ambient and chilled storage, respectively), dietary fibre content, isoflavone content, and aroma. All values were expressed as g compound per 100 g dried okara (DW, dry weight).

Results

Probiotic viability

Probiotic viability, yeast count and pH in different fermented treatments after 8 weeks of storage at 25°C and 5°C were tabulated and the results are as shown in Table 3.

Treatment	Probiotic count (log CFU/mL)	Yeast count (log CFY/mL)	pH
Storage at 25°C			
All_1	7.53±0.41	6.32±0.05	3.69±0.01
All_2	7.21±0.16	6.83±0.20	3.69±0.01
All_3	7.14±0.16	7.02±0.35	3.69±0.06
All_4	6.41±0.49	6.73±0.24	3.13±0.01
All_5	6.87±0.35	5.99±0.14	3.23±0.02
All_6	7.20±0.20	6.07±0.14	3.24±0.02
All_7	6.48±0.02	6.53±0.05	3.20±0.04
All_8	7.18±0.44	5.92±0.49	3.26±0.05
Storage at 5°C			
All_1	8.57±0.42	5.89±0.27	3.66±0.12
All_2	8.46±0.47	6.50±0.37	3.69±0.14
All_3	8.05±0.69	6.22±0.49	3.59±0.09
All_4	7.55±0.38	6.18±0.45	3.09±0.05
All_5	8.33±0.80	5.74±0.42	3.18±0.07
All_6	8.56±0.78	5.73±0.74	3.21±0.05
All_7	8.38±0.73	6.17±0.46	3.17±0.08
All_8	7.93±0.05	6.10±0.71	3.28±0.08

Table 3: Probiotic and yeast counts and pH after 8 weeks storage at 25°C and 5°C

In order to investigate the effect of the addition of probiotics and yeast on the probiotic cell viability, a comparison of cell viability after 8 weeks was also conducted between selected All, MonoP and MonoY treatments as shown in Table 4.

Treatment	Probiotic count (log CFU/mL)	Yeast count (log CFY/mL)	pH
Storage at 25°C			
All_1	7.53±0.41	6.32±0.05	3.69±0.01
MonoP_1	7.27±0.13	-	3.20±0.30
MonoY_1	-	6.69±0.20	4.18±0.53
All_3	7.14±0.16	7.02±0.35	3.69±0.06
MonoP_3	6.56±0.35	-	3.24±0.23
MonoY_3	-	5.00±0.00	4.10±0.73
Storage at 5°C			
All_1	8.57±0.42	5.89±0.27	3.66±0.12
MonoP_1	8.23±0.03	-	3.25±0.24
MonoY_1	-	6.82±0.12	4.40±0.68
All_3	8.05±0.69	6.22±0.49	3.59±0.09
MonoP_3	7.18±0.20	-	3.32±0.15
MonoY_3	-	7.10±0.10	4.63±0.50

Table 4: Probiotic and yeast counts and pH after 8 weeks storage at 25°C and 5°C

It can be seen from Table 4 that the presence of yeast was crucial for the viability of probiotics in the okara-based probiotic beverage during chilled and ambient storage, as reflected in viable probiotic count in the co-cultures and mono-cultures. After 8 weeks at 25°C, the decrease in viable probiotic was lower in the All co-cultures compared to the corresponding MonoP mono-cultures.

When stored for 8 weeks at 5°C, the viable probiotic count actually increased in the All co-cultures, compared to the decrease observed in the corresponding MonoP mono-cultures. Therefore, the presence of yeast seemed to enhance the probiotic viability in these combinations. Further, the yeast may have prevented the pH from falling too much in the All co-cultures, thereby buffering the probiotics against pH stress.

Dietary fibre

The changes in the composition of the dietary fibres in the okara was investigated. In particular, the effect of heat on soluble dietary fibres (SDF) was investigated. The results are as shown in Figure 4.

As can be seen from Figure 4, heating significantly increased the amount of soluble fibre in all carbohydrase-treated treatments. Further, between the carbohydrases, PX and VZ were more effective than CC in increasing the amount of SDF.

The effect of the IDF and SDF content during storage of the okara-based beverage was also tested and the results are provided in Table 5.

Conditions	Treatment	IDF (g/ 100g DW)	SDF (g/ 100g DW)
	CC-heat	69.8±7.4	9.6±0.9
After 8 weeks at 5°C	All_3	69.7±7.4	6.8±1.3
After 8 weeks at 25°C	All_3	69.4±8.2	10.7±0.5
After 8 weeks at 25°C	MonoP_3	72.9±10.4	7.8±0.1
After 8 weeks at 25°C	MonoY_3	60.6±11.5	6.8±1.1
	VZ-heat	65.7±9.9	11.9±0.5
After 8 weeks at 5°C	All_6	65.1±4.1	7.3±0.3
After 8 weeks at 25°C	All_6	60.8±0.6	15.7±1.6
After 8 weeks at 25°C	Co_6	82.6±1.1	5.4±0.2

Table 5: Changes in IDF and SDF after 8 weeks storage at 25°C and 5°C

As seen in Table 5, after 8 weeks of storage at 5°C, the okara-based probiotic beverage still contained at least 6.8 – 7.3 g SDF/100 g DW (in All 3 and All 6, treatments), while at storage at 25°C, the beverage contained at least 10.7 – 15.7 g SDF/100 g DW. However, the amount of SDF may decrease during fermentation and storage due to its utilization by microorganisms within the beverage. This can be seen from Table 5 where in the treatments containing CC (treatments CC-heat, All_3, MonoP_3 and MonoY_3) the SDF remaining after carbohydrase treatment generally decreased after 8 weeks. Further, storing the okara probiotic beverage at chilled temperature seemed to reduce the SDF to a greater extent than when stored at ambient temperature. This is reflected by the lower amount of SDF in both All 3 and All 6 treatments stored at 5°C compared to their corresponding counterpart stored at 25°C after 8 weeks.

Additionally, carbohydrase is key to reducing IDF and increasing SDF, as reflected by the difference in IDF and SDF between Co_6 and All_6 treatments. Thus, it can be seen that while the inoculated microorganisms may break down some dietary fibre over time, the extent will not be as extensive as that of carbohydrase pre-treatment.

Isoflavone

Isoflavone aglycones can reduce plasma cholesterol and improve carbohydrate metabolism in animal models. Following carbohydrase treatment, most isoflavone glycosides (daidzin, genistin and glycitin) were converted into their aglycone forms (daidzein, genistein and glycitein, respectively), as can be seen in Figure 5. This hydrolysis was catalysed by endo- β -glycosidases in the carbohydrase.

Heating led to a slight decrease in isoflavone aglycones but also increased the amount of isoflavone glycosides. Fermentation further reduced the amount of isoflavone glycosides (comparing treatments CC-heat, All_2 and Co_2).

The change in the isoflavone content following storage after 8 weeks was also observed. The results are as shown in Table 6.

Treatment	Isoflavone glycosides ($\mu\text{g/g DW}$)	Isoflavone aglycones ($\mu\text{g/g DW}$)
All_2 (After fermentation)	9.5 $\pm$ 2.1	356.0 $\pm$ 53.2
Stored at 5°C for 8 weeks		
All_1	4.7 $\pm$ 0.5	323.1 $\pm$ 14.1
All_2	ND	332.7 $\pm$ 64.6
Stored at 25°C for 8 weeks		
All_1	ND	349.8 $\pm$ 50.3
All_2	ND	407.0 $\pm$ 11.6
MonoP_1	ND	343.9 $\pm$ 19.3
MonoY_1	ND	506.7 $\pm$ 15.5

Table 6: Changes in isoflavone content after 8 weeks storage at 25°C and 5°C

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From Table 6, it can be seen that after 8 weeks of storage at 5°C, the okara-based probiotic beverage (combinations All_1 and All_2) contained at least 350 μg isoflavone aglycones/g DW. When stored at 25°C for 8 weeks, the amount of isoflavone aglycones was slightly higher. Accordingly, it is likely that the yeast produced glycosidases that converted isoflavone glycosides into aglycones over time, as evident

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from the higher amount of isoflavone aglycones in MonoY_1 treatment compared to All_1 treatment.

Volatiles

The changes in different class of volatiles are as shown in Table 7. The unfermented
5 okara contained mainly aldehydes, which have a green, grassy off-odour. These aldehydes were likely formed from the degradation of soy polyunsaturated fatty acids during cold grinding. The enzyme treatment had likely broken down the okara cell wall and released more unsaturated fatty acids which were spontaneously oxidised.

Fermentation led to rather different volatile profiles. Adding probiotic only (e.g.
10 MonoP_3 and MonoP_5) in the okara slurry resulted in mainly acids and alcohols, leading to a sour, rancid odour. Moreover, some probiotics, such as that in MonoP_5, may produce more acids than other probiotics, such as that in MonoP_3. Adding yeasts in the okara slurry changed the aroma profile to a greater extent – however, only selected yeasts did that. For example, only MonoY_3 and MonoY_5 contained
15 greater amounts of esters, while MonoY_1 and MonoY_7 did not increase the amount of esters.

The importance of a suitable yeast in okara probiotic beverage is reflected in the aroma profiles of All_1 and All_3. In All_1, as the yeast used (same yeast as in MonoY_1) did not produce many esters, the main class of volatiles was alcohol. Comparatively, in
20 All_3, as the yeast used (same yeast as in MonoY_3) produced a high amount of ester, the main class of volatiles was esters, and the okara-based probiotic product smelled pleasant and fruity.

The esters included isoamyl acetate (banana-like odour), hexyl acetate (fruity odour) and 2-phenylethyl acetate (floral, rose-like odour), among others. Due to the great
25 amounts of esters formed, the EYL coculture treatment still smelled fruity despite the fair amount of volatile acids present.

	CC-heat		PX-heat		VZ-heat	
	µg/g DW	(%)	µg/g DW	(%)	µg/g DW	(%)
Acids	0.57 ± 0.08	(12.3)	1.28 ± 0.15	(9.8)	1.03 ± 0.18	(20.1)
Alcohols	0.67 ± 0.06	(14.4)	0.93 ± 0.03	(7.2)	0.56 ± 0.08	(10.8)
Aldehydes	3.36 ± 0.08	(72.3)	10.80 ± 0.82	(82.9)	3.56 ± 0.44	(69.1)
Esters	0.04 ± 0.01	(0.9)	0.02 ± 0.00	(0.1)	ND	(0.00)
Ketones	ND	(0.00)	ND	(0.00)	ND	(0.00)

	MonoY1		MonoY3		MonoY5		MonoY7	
	µg/g DW	(%)	µg/g DW	(%)	µg/g DW	(%)	µg/g DW	(%)
Acids	2.04 ± 0.19	(12.5)	3.51 ± 0.62	(4.0)	8.05 ± 1.12	(6.5)	13.36 ± 1.61	(37.8)
Alcohols	11.26 ± 0.93	(69.4)	14.10 ± 2.11	(16.1)	19.11 ± 3.97	(15.5)	17.43 ± 0.00	(49.3)
Aldehydes	0.25 ± 0.02	(1.5)	1.18 ± 0.29	(1.3)	5.43 ± 0.24	(4.4)	3.71 ± 0.34	(10.5)
Esters	2.69 ± 0.17	(16.6)	68.82 ± 6.10	(78.6)	90.87 ± 7.86	(73.6)	0.84 ± 0.00	(2.4)
Ketones	ND	(0.00)	ND	(0.00)	ND	(0.00)	ND	(0.00)

	MonoP3		MonoP5		All 1		All 3	
	µg/g DW	(%)	µg/g DW	(%)	µg/g DW	(%)	µg/g DW	(%)
Acids	2.23 ± 0.41	(22.1)	5.27 ± 1.26	(32.6)	1.69 ± 0.17	(15.2)	5.21 ± 0.74	(7.8)
Alcohols	6.22 ± 0.50	(61.5)	6.02 ± 0.00	(37.2)	7.60 ± 0.33	(68.2)	1.52 ± 0.04	(2.3)
Aldehydes	0.39 ± 0.05	(3.9)	4.16 ± 0.71	(25.7)	1.57 ± 0.23	(14.1)	1.34 ± 0.14	(2.0)
Esters	0.90 ± 0.25	(8.9)	0.06 ± 0.00	(0.4)	0.28 ± 0.05	(2.5)	59.12 ± 9.34	(88.0)
Ketones	0.37 ± 0.06	(3.6)	0.67 ± 0.16	(4.1)	ND	(0.00)	ND	(0.00)

ND = Not detected. % = expressed as a percentage of the total amount of volatiles in sample.

Table 7: Changes in major classes of volatiles in selected treatments

Overall, treatments All_3 and All_5 resulted in higher amounts of esters being comprised in the okara-based beverage. Accordingly, the changes in the volatile profile of the okara-based beverages formed from treatments All_3 and All_5 were investigated. The results are as shown in Table 8.

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After fermentation								
	All 3				All 5			
	µg/g DW			(%)	µg/g DW			(%)
Acids	5.21	±	0.74	(7.8)	1.48	±	0.15	(7.6)
Alcohols	1.52	±	0.04	(2.3)	5.70	±	0.73	(29.3)
Aldehydes	1.34	±	0.14	(2.0)	0.99	±	0.18	(5.1)
Esters	59.12	±	9.34	(88.0)	11.32	±	5.39	(58.1)
Ketones	ND			(0.0)	ND			(0.0)
Stored at 5 °C for 8 weeks								
	All 3				All 5			
	µg/g DW			(%)	µg/g DW			(%)
Acids	2.08	±	0.48	(8.9)	2.67	±	0.22	(6.8)
Alcohols	6.88	±	0.00	(29.6)	6.03	±	0.56	(15.3)
Aldehydes	1.63	±	0.48	(7.0)	1.57	±	0.21	(4.0)
Esters	12.65	±	2.03	(54.4)	29.11	±	3.96	(73.9)
Ketones	ND			(0.0)	ND			(0.0)
Stored at 25 °C for 8 weeks								
	All 3				All 5			
	µg/g DW			(%)	µg/g DW			(%)
Acids	3.85	±	0.68	(5.2)	5.43	±	0.55	(6.3)
Alcohols	0.88	±	0.18	(1.2)	1.02	±	0.21	(1.2)
Aldehydes	1.15	±	0.06	(1.5)	1.04	±	0.14	(1.2)
Esters	68.44	±	8.18	(92.1)	78.75	±	6.03	(91.3)
Ketones	ND			(0.0)	ND			(0.0)

ND = not detected. % = expressed as a percentage of the total amount of volatiles in sample.

Table 8: Changes in major classes of volatiles after 8 weeks storage at 25°C and 5°C

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After 8 weeks of storage at 5°C, the okara probiotic beverage still retained at least 20% of the original amount of esters, as can be seen in Table 8, and esters still remained as

the major group of volatiles in the okara-based probiotic beverage. The drop in the amount of esters may be due to the hydrolysis of esters into alcohols and acids (some of which could have been catabolized by the microorganisms), while some may have evaporated over time.

- 5 On the other hand, after 8 weeks of storage at 25°C, the okara-based probiotic beverage still retained at least 100% of the original amount of esters. In fact, the yeast continued to grow and maintained higher populations over time, resulting in an increase in esters. Therefore, esters remained as the major class of volatile in both All_3 and All_5 treatments following storage.

10 *Conclusion*

It can therefore be seen that the okara-based probiotic beverage contains various health beneficial components such as soluble fibres, probiotics, isoflavone aglycones, a pleasant aroma due to the high amount of esters and stable ambient shelf-life for up to 8 weeks with viable probiotic count remaining at $\geq 7 \log$ CFU/mL.

- 15 When stored at 5°C for 8 weeks, compared to the original product, the final okara-based probiotic beverage retained at least 70% of SDF, at least 90% of isoflavone aglycones and at least 20% of esters. When stored at 25°C for 8 weeks, compared to the original product, the final okara-based probiotic beverage retained almost the same amount of SDF, isoflavone aglycones and esters.