Hort Innovation

Final Report

ProbiSafe – Development of biocontrol agents to inhibit pathogen growth

Project leader:

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Delivery partner:

Uniquest Pty Ltd and University of Queensland

Project code:

VG16005

Project:

Probisafe – Development of biocontrol agents to inhibit pathogen growth VG16005

Disclaimer:

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Content

Summary

Recent *Salmonella* outbreaks in Australia associated with salad leaves and sprouts in 2016 had a significant impact on the vegetable industry. Disinfection methods incorporating chlorine, ozone, organic acids and peracetic acid are often applied to fresh vegetables, however, these disinfectants have limited antimicrobial efficacy, potential toxicity and side effects on the sensory properties of the products. This project developed and verified new biocontrol agents (*Probisafe*) to inhibit the growth of *Salmonella* on salad products. The other part of this project explored commercial probiotic delivery using vegetables.

Probisafe strains were tested for inhibiting *Salmonella* growth on fresh-cut iceberg lettuce at different conditions. The growth of *Salmonella* was found to vary with the application methods of *Salmonella* suspension in lettuce by spotting or soaking. The counts of lactic acid bacteria (LAB) and *Salmonella* remained relatively unchanged or even slightly decreased in the spotting samples whereas the growth of more than 1 log over 7 days was observed in the soaking samples. The increased initial concentrations of *Probisafe* strains led to the decrease in the pH of lettuce which adversely affected lettuce appearance and firmness. Initial *Probisafe* levels of 10^8 CFU/g were shown to be effective in inhibiting *Salmonella* without compromising the sensory quality of lettuce. Three most promising *Probisafe* strains (*Leuconostoc mesenteroides* 109, 774 and 845) showed significant inhibition of *Salmonella* in shredded iceberg lettuce stored at 12°C. The analysis of the whole genome sequence data revealed that the majority of highly specific virulence genes were not found in the genomes of *Probisafe* strains and the possibility of antibiotic resistance transfer from the *Probisafe* strains to other bacteria is not likely.

Thirteen commercial probiotic strains obtained from two different culture supply companies were evaluated for their survival in baby spinach. Additionally, another three commercial probiotic strains (*Lactobacillus rhamnosus* GG, *Lactobacillus acidophilus* NCFM and *Lactobacillus plantarum* 299v) from the UQ culture collection were also tested. Most of these probiotic strains showed excellent survival in spinach leaves, suggesting that probiotic delivery using commercial bagged salads is feasible. Levels of > 1 billion live probiotic cells in a 100 g serve of spinach appear likely. Survival of probiotics in baby spinach was also tested under adverse conditions. The addition of various salad dressings to probiotic-supplemented spinach did not adversely affect the viability of the probiotics. Probiotic strains tested also survived well in the presence of simulated gastric and intestinal juices. Sensory trials performed by 40 un-trained panellists had demonstrated that the quality of spinach leaves was not adversely affected by the addition of commercial probiotics. Two of Australia's largest fresh-cut salad producers have shown great interest in probiotic vegetables and are keen to start trials of probiotic application in their salad products. A new category of salad products is anticipated to be released to market in the near future.

Public Summary

Recent *Salmonella* outbreaks in Australia associated with salad leaves and sprouts in 2016 had a significant impact on the vegetable industry. Disinfection methods are often applied to fresh vegetables, however, these disinfectants have limited antimicrobial efficacy and are undesirable from the consumer perspective. This project developed and verified new naturally present harmless bacteria, called *Probisafe*, which can block the growth of *Salmonella* on salad products. The other part of this project involved the development of commercial probiotic bacteria containing vegetables as an alternative to dairy or capsule/tablet based probiotic products.

Probisafe bacteria were tested for inhibiting *Salmonella* growth on fresh-cut iceberg lettuce at different conditions. Initial *Probisafe* levels of 100 million bacteria per gram lettuce did not compromise the quality of lettuce when stored under normal refrigerated conditions. However upon storage at slightly higher temperatures, the increased initial concentrations of *Probisafe* strains led to adverse effects on the lettuce appearance and firmness, but also significantly inhibited *Salmonella* growth. Using new DNA sequencing technology all the genes in the *Probisafe* bacteria were analysed and found to be safe, with no genes for antibiotic resistance or which allow infection found. Therefore *Probisafe* bacteria can be useful in ensuring safe leafy green products during potential storage temperature abuse.

Sixteen commercial probiotic bacteria were evaluated for their survival in baby spinach leaves. Most of these probiotic strains showed excellent survival in spinach leaves, suggesting that probiotic delivery using commercial bagged salads is feasible. Levels of more than 1 billion live probiotic cells in a 100 g serve of spinach appear possible. The addition of various salad dressings to probiotic-supplemented spinach did not significantly reduce the number of probiotics. Probiotic bacteria tested also survived well during laboratory simulated digestion (stomach and intestine) trials. Taste testing trials performed by 40 human volunteers demonstrated that the quality of spinach leaves was not adversely affected by the addition of commercial probiotics. A new category of probiotic containing salad products has the potential to provide additional health-promoting properties to consumers.

Keywords

Probisafe, bacteria; fresh salads; anti-*Salmonella*; food safety; iceberg lettuce; baby spinach; probiotics; health; product development

Introduction

Fresh produce provides excellent nutrients, vitamins and fibre and packaged leafy greens in particular have become very popular with consumers. Since fresh produce undergoes minimal processing and is in most cases eaten raw, it is inherently risky from a food safety stand-point. Pathogens such as *Salmonella*, *Escherichia coli* and *Listeria monocytogenes* have caused outbreaks in leafy greens (lettuce and spinach), tomatoes, cucumbers and sprouts around the world. Two recent large outbreaks in Australia from *Salmonella*-contaminated leafy greens and sprouts resulted in over 500 illnesses in Victoria and South Australia and is a significant issue for the vegetable industry.

Pathogens can contaminate vegetables either pre- or post-harvest. Pre-harvest sources can include soil, faeces, irrigation water, dust, insects, inadequately composted manure, wild or domestic animals and human handling (Olaimat and Holley, 2012). Post-harvest sources include harvesting equipment, transport containers and equipment, insects, dust, wash water, ice, processing equipment and human handling (Beuchat, 2002). Controlling contamination is the best way to prevent foodborne illness, however additional controls which either kill or inhibit the growth of low levels of contaminating pathogenic bacteria on vegetables would also be expected to improve vegetable safety.

Post-harvest washing of vegetables in most cases with chlorine results in a reduction in the total number of bacteria, including pathogens, by approximately 1-2-log (10-100-fold kill). The effectiveness of this treatment however can be compromised by pathogen internalisation into the leaf tissue and biofilm formation and chlorine residues remain on the vegetable which is undesirable from a consumer stand-point. Other methods have been examined for reducing pathogen numbers on vegetables include irradiation, ozone-enriched water, organic acids, bacteriophages (Olaimat and Holley, 2012) which have limitations that range from consumer acceptance (irradiation), oxidation of food (ozone), residues (organic acids) and short-lived activity (bacteriophages). Antagonistic or biocontrol bacteria have also been explored for pathogen inhibitory activity on vegetables (Cooley et al., 2006; Scolari and Vescovo, 2004; Trias, Badosa, et al., 2008; Trias, Baneras, et al., 2008; Trias et al., 2009). The application of 'generally regarded as safe' (GRAS) biocontrol bacteria which were originally isolated from vegetables to control pathogens in vegetables provides a natural approach where the antimicrobial substance is produced *in situ* on the vegetable surface.

In a previous HAL funded project in 2009-2011 (VG09075) we investigated biocontrol strategies to inhibit foodborne pathogens (*Listeria monocytogenes* and *Salmonella* Typhimurium) on salad vegetables. Around 900 naturally occurring harmless lactic acid bacteria (LAB) were selected from a wide variety of vegetables and fruits in the field and screened for antimicrobial activity against *Listeria* and *Salmonella*. Sixty-nine LAB had activity against one or both pathogens. Some of these LAB were able to reduce *Listeria* and *Salmonella* growth by up to 99.9% after 7 days of storage. Through the use of a combination of chlorine and LAB treatment, *Listeria* were able to be completely eliminated to undetectable levels over the 7 day storage period on cut lettuce products. No negative spoilage-related effects of LAB addition to lettuce were noticed. In this project we plan to confirm the activity of these biocontrol LAB, termed *Probisafe*, and identify optimal conditions for their application at lab-scale and in industry trials.

The other part of this project will explore the potential of vegetables to deliver health-promoting probiotics. Foods with added probiotic microorganisms have attracted

significant attention of both consumers and the food industry. Dairy products (e.g. yoghurts) are currently the most common vehicle for delivering probiotics in the market, however probiotic containing vegetables have the potential as an alternative option for consumers who are intolerant to dairy products or require low-cholesterol diets. This project will therefore also evaluate commercial probiotic delivery using vegetables.

Methodology

1. Development of natural biocontrol bacteria (termed *Probisafe*) for industry application to control pathogen growth on vegetables

1.1. Laboratory trials

1.1.1. Bacterial strains and culture conditions

The *Probisafe* bacterial strains used in this study were isolated from fruits and vegetables in a previous HAL funded project (VG09075 completed in 2011). Bacterial strains and their sources are listed in Table 1.

Liquid cultures of LAB were grown in de Man Rogosa Sharpe (MRS) broth (Oxoid, Basingstoke, UK) and incubated overnight in tubes with minimal headspace without agitation at 30 ˚C. *Salmonella* Typhimurium ATCC 14028, which was used as an indicator strain of antimicrobial activity of *Probisafe* bacteria, was aerobically cultured in Brain Heart Infusion (BHI) medium (Oxoid, Basingstoke, UK) at 37 ˚C. The enumeration of LAB and *S*. Typhimurium populations was carried out on MRS and XLT4 agar (Oxoid), respectively. In addition, a double layer agar was developed for *S*. Typhimurium enumeration and compared with a resuscitation method for recovering injured *Salmonella* cells as described in Section 1.1 in Appendix A.

Table 1 Biocontrol bacterial strains used

Isolate No.	Closest match	Isolation source
22	*Weissella cibaria*	Mixed salad
34	*Weissella cibaria*	Parley
37	*Weissella confusa*	Cos lettuce
44	*Weissella confusa*	Cos lettuce
109	*Leuconostoc mesenteroides*	Iceberg lettuce
140	*Weissella cibaria*	Sweet potato leaves
156	*Leuconostoc pseudomesenteroides*	Sweet potato leaves
493	*Weissella soli*	Baby cos leaves
680	*Lactococcus lactis*	Chinese cabbage
684	*Leuconostoc mesenteroides*	Green chilli
733	*Leuconostoc holzapfelii*	Honeydew melon
744	*Weissella confusa*	Rock melon
749	*Weissella confusa*	Rock melon
752	*Weissella cibaria*	Watermelon
754	*Leuconostoc pseudomesenteroides*	Watermelon
757	*Lactococcus raffinolactis*	Watermelon
758	*Weissella cibaria*	Watermelon
774	*Leuconostoc mesenteroides*	Pawpaw
789	*Lactococcus lactis*	Pawpaw
817	*Weissella cibaria*	Green bean
818	*Leuconostoc lactis*	String bean
820	*Leuconostoc holzapfelii*	Sugarsnap peas
824	*Lactococcus lactis*	Beetroot
838	*Leuconostoc mesenteroides*	Parsnips
845	*Leuconostoc mesenteroides*	Parsnips

38	*Lactococcus lactis*	Baby spinach. No inhibition against *S*. Typhimurium

1.1.2. Confirmation of anti-*Salmonella* activities of biocontrol lactic acid bacteria

Bacterial strains showed preliminary inhibition against *Salmonella* Typhimurium in the initial screening were selected for confirmation. The isolates that displayed no inhibitory activity were used as negative control. An agar overlay assay method was used for detection of antimicrobial activity of LAB isolates against *Salmonella* Typhimurium ATCC 14028. LAB and *S*. Typhimurium are cultured as previously described in Section 1.1.1. BHI agar plates (1.4 % agar) were overlaid with 10 mL of soft MRS agar (0.7 % agar) containing 10^6 CFU/mL of S. Typhimurium. The plates were dried in a biosafety cabinet for 30 min before spotting overnight cultures of LAB (10 µl) on the surface of dried agar. The plates were then dried and incubated aerobically at 30 °C for 24 h and the antimicrobial activity was determined by observation of clearing zones of inhibition around LAB spots.

1.1.3. Attachment of *Probisafe* isolates on shredded iceberg lettuce under different treatment times

Two selected LAB isolates (752 and 38) were cultured anaerobically in MRS broth at 30 ˚C for 24 h to reach stationary phase. The cultures were harvested by centrifugation at 5000 × *g* for 10 min, washed twice with 0.85 % saline solution and then resuspended in the same solution prior to being inoculated into shredded iceberg lettuce for different treatment times (1, 5, 15 and 30 min). Lettuce samples were incubated at 8 ˚C for 7 days. The populations of biocontrol strains were determined on days 0, 3 and 7. Details on the methodology can be found in Appendix A (Section 1.2).

1.1.4. Effects of different initial concentrations of *Probisafe* strains on lettuce quality

To evaluate the effects of initial concentrations of biocontrol strains on the quality of lettuce, two selected LAB (109 and 752) were individually inoculated into shredded iceberg lettuce at three different levels (10^7, 10^8 and 10^9 CFU/g shredded lettuce). LAB were cultured as previously described and the inoculation of LAB into lettuce is described in Section 1.3 in Appendix A. The inoculated lettuce was stored at 8 ˚C for 7 days. The populations of LAB were counted on days 0, 3 and 7.

1.1.5. Inhibition of *S*. Typhimurium growth by *Probisafe* strains in ready-to-eat whole and cut leafy vegetables

To evaluate the effectiveness of *Probisafe* strains on inhibition of *S.* Typhimurium growth in leafy vegetables, five selected biocontrol strains (22, 44, 733, 752 and 838) were individually inoculated onto whole leave salads (baby spinach and rocket), shredded iceberg lettuce and shredded cabbage and carrot. *L. lactis* 38 showed no inhibition activity against *S.* Typhimurium was used as a negative control strain. The inoculation of *S*. Typhimurium ATCC 14028 and biocontrol LAB strains into lettuce was described in Section 1.4 in Appendix A. The populations of biocontrol strains and *S.* Typhimurium were determined on days 0, 3 and 7.

1.1.6. Preparation of shredded iceberg lettuce using spin and no spin methods

In our previous experiments, after soaking the shredded lettuce pieces in a bacterial suspension, the extra liquid was removed by decanting or pipetting. However, in the industrial setting there is a spinning step to remove extra liquid from the salad leaves before packaging, so there was concern that our lettuce might have extra moisture,

leading to results not representative of commercial lettuce. Therefore, we simulated the industrial spinning process by spinning the shredded lettuce in a home-use salad spinner for 2 min and compared the moisture content and water activity between spun and not spun lettuce samples. Shredded lettuce pieces were prepared using spin and no spin methods as described in Appendix A (Section 1.5).

1.1.7. Application of *Salmonella* in shredded lettuce by spotting vs soaking

We have applied *Salmonella* to lettuce either by adding 10 mL of cell suspension and shaking for even distribution or soaking in 500 mL of cell suspension. These methods will result in aerosol (former) and large volumes of *Salmonella* waste (latter). In an attempt order to further minimise risk when carrying out experiments with *Salmonella*, we tested whether applying *Salmonella* by spotting a small volume of cell suspension onto lettuce as described below is a good alternative method.

In our previous experiments, lettuce was treated first with *Salmonella*, and then with LAB. The reasoning for this order of application is to simulate real life situations in which *Salmonella* contamination is most likely to take place in the farm, prior to deliberate addition of biocontrol strains. However, in order to apply *Salmonella* with the spotting method, the spotting has to take place after LAB treatment in order to prevent washing away the *Salmonella* cells in the LAB soak. A total of 200 µL of *Salmonella* suspension was applied at various points on 20 g of lettuce. Another batch of samples in which *Salmonella* was applied by soaking as described in Section 1.6 in Appendix A was prepared, and the growth of *Salmonella* and LAB in the two types of samples were monitored.

1.1.8. Inhibition of *Salmonella* in shredded iceberg lettuce by *Probisafe* strains at different storage temperatures

The effects of recommended (≤ 5 °C) and abuse storage temperatures on the growth and survival of biocontrol strains and *Salmonella* on minimally processing vegetables were observed at 4 and 12 °C. A storage temperature of 4 °C was chosen to stimulate properly refrigerated conditions whereas a storage temperature of 12 °C was selected to stimulate abusive temperatures frequently occurring during marketing and distribution of fresh-cut produce (Luo et al., 2010). The abusive temperatures may result in the proliferation of pathogens to populations likely to represent a food safety problem and hence the effects of *Probisafe* strains on inhibiting *Salmonella* in fresh-cut lettuce would be evaluated under conditions close to reality. The methodology in the inoculation of S. Typhimurium and *Probisafe* bacteria into shredded lettuce is shown in Section 1.7 in Appendix A.

1.2. Industry trial

1.2.1. Development of food grade media to grow *Probisafe* bacterial strains

Probisafe strains are normally grown in our lab in non-food grade growth media which is not suitable for application in a food production facility. Therefore we have designed and tested several new growth media made using food grade ingredients for growing *Probisafe*. Four different media were made as below in Table 2.

Table 2 Food grade growth media formulations developed and tested for growing *Probisafe* strains

Formulation	Ingredients	Percentage (%)
A	Glucose	1.33

	Yeast Extract	1.33
B	Glucose	1.33
	Yeast extract	1.33
	Potassium phosphate	0.27
	Tween 80	0.67
	Sodium sulfate	0.07
C	Glucose	1.33
	Yeast extract	1.33
	Sodium citrate	0.27
	Tween 80	0.67
	Sodium sulfate	0.07
D	Glucose	1.33
	Yeast Extract	1.33
	Potassium phosphate	0.27

1.2.2. Industry trials with the three most promising *Probisafe* strains

Three most promising *Probisafe* strains including 109, 774 and 845 were anaerobically grown in the food grade medium (formula D) for 40 h at 30 °C. The cultures of *Probisafe* bacteria were harvested and washed with saline solution as previously described in Section 1.1.3. The preparation of *Probisafe* strain cultures were carried out in food grade laboratories at The University of Queensland and then the cultures were transported in an esky to the fresh-cut producer within 1 h. Shredded iceberg lettuce was prepared and supplied by the industry partner. The inoculation of *Probisafe* strains into shredded iceberg lettuce and their enumeration were performed as described in Section 1.6 in Appendix A.

1.3. Safety evaluation and whole genome sequences of LAB strains

1.3.1. Biogenic amines

Lettuce samples were prepared with three different species of candidate biocontrol LAB strains (*L. mesenteroides* 109, *W. confusa* 744 and *W. cibaria* 752) and packaged under modified atmosphere packaging (MAP) which contained 4% O2, 12% CO2 and balanced by N2. After storage at 8 °C for 5 days, the samples were sent to Symbio Laboratories (Brisbane, QLD) for detection of four types of biogenic amines including β-phenylethylamine, putrescine, cadaverine and histamine which are commonly found in food.

1.3.2. Whole genome sequences of LAB strains

Twelve strains of candidate biocontrol LAB strains belonging to the genera *Weissella* and *Leuconostoc* were submitted to Macrogen (Seoul, Korea) for whole genome sequencing using Illumina HiSeq2500 (Table 3). The reads were assembled into draft genomes using Velvet (Zerbino and Birney, 2008) and annotated with RAST (Aziz et al., 2008). A survey of genes linked to virulence and antibiotic resistance was performed on the genome sequences of these 12 strains and 3 nisin-positive *Lactococcus lactis* strains isolated from fruits and vegetables that were previously sequenced (strain no. 21, 417 and 537).

Table 3 Strains sent for whole genome sequencing

Species	Strain no.
Weissella cibaria	22
Weissella confusa	44
Leuconostoc mesenteroides	109

Leuconostoc mesenteroides	156
Weissella soli	498
Leuconostoc mesenteroides	684
Leuconostoc holzapfelii	733
Weissella confusa	744
Weissella cibaria	752
Weissella cibaria	758
Leuconostoc lactis	824
Leuconostoc mesenteroides	838

2. Evaluation of commercial probiotic delivery using vegetables

2.1. Laboratory trials

2.1.1. Bacterial growth conditions and enumeration of commercial probiotics in baby spinach

Probiotic strains in the form of freeze-dried powder provided by two different companies were tested: 3 strains from company A and 10 strains from company B. Probiotic powder was rehydrated in tap water and their suspension was used to soak baby spinach. Bacterial strains from the UQ culture collection were grown in MRS medium at 37 °C. Liquid cultures were incubated overnight without agitation and with little headspace, and agar plates were incubated anaerobically for 48 h. Enumeration of probiotic bacteria was carried out by preparing ten-fold dilutions in 0.1% peptone water (bacteriological peptone, Oxoid) and spread plating on MRS agar.

Commercial probiotic suspension was added into baby spinach by soaking method to reach an initial counts of 10^7-10^9 CFU/g as described in Section 2.1 in Appendix A. Enumeration by spread plating was done on days 0, 3 and 7 of storage.

2.1.2. Survival of probiotics in baby spinach in presence of salad dressing, simulated gastric and intestinal juice

Stocks of six probiotic strains were prepared as described in Section 2.2 in Appendix A: 3 strains from company A (A-1, A-2, A-3) and 3 that were part of the UQ collection: *Lactobacillus acidophilus* NCFM, *Lactobacillus plantarum* 299v and *Lactobacillus rhamnosus* GG. These probiotic strains were tested in the presence of three types of salad dressing: French, Italian and Balsamic (Praise, NSW, Australia) while five strains with the exception of A-3 were tested in the presence of simulated gastric and intestinal juice. Details on the methodology are described in Sections 2.2.1 and 2.2.2 in Appendix A.

2.1.3. Sensory evaluation of baby spinach added commercial probiotics

Ready-to-eat baby spinach leaves were purchased from Coles. Two commercial probiotic strains in the form of freeze-dried powder were added into baby spinach at an initial concentration of 10^7-10^9 CFU/g. A control sample without probiotic addition was also prepared. The details in the addition of probiotics into baby spinach was described in Section 2.1 in Appendix A. Enumeration of probiotic counts was conducted on days 0, 3 and 7.

Before the sensory trial, probiotic spinach samples prepared in the same manner as described above were sent to Symbio Laboratories (Brisbane, QLD, Australia) for analysing microbiological safety to ensure they were free from pathogens. The recruitment for the sensory panel, the method of sensory evaluation and the sensory evaluation form were described in Section 2.5 in Appendix A.

2.2. Industry trial

Two probiotic strains (A-2 and A-3) from company A and one strain (B-8) from company B were selected for the industry trial due to their good survival in baby spinach during laboratory trials at The University of Queensland. Baby spinach leaves were prepared and supplied by the industry partner. The addition of probiotic bacteria into baby spinach at the factory of the fresh-cut producer was carried out as descried in Section 2.1 in Appendix A.

Outputs

1. Press releases were published on UQ website and picked up by several media outlets.

- ***'Probiotics' on salad greens could make them safer, healthier*** (Published on 14th November, 2016). http://www.uq.edu.au/news/article/2016/11/probiotics%E2%80%99-salad-greens-could-make-them-safer-healthier
- ***'Probiotics in salads probed as solution to* Salmonella *outbreaks in bagged mixes'*** (Published on 5th February, 2017). http://www.abc.net.au/news/2017-02-05/bacteria-probisafe-bagged-salads-salmonella-listeria/8237000

2. A/Prof Mark Turner was interviewed by ABC News and the episode was published on their channel on 5th February, 2017. http://www.abc.net.au/news/2017-02-05/bacteria-probisafe-bagged-salads-salmonella-listeria/8237000

3. A/Prof Mark Turner was interviewed by radio stations (AAP, 3AW, 2GB Sydney and ABC Rural) in November 2016 and February 2017.

4. Various online media stories have appeared on this research project – links below:

- http://www.sbs.com.au/news/article/2016/11/15/good-bacteria-fight-salmonella-cases
- http://www.foodanddrinkbusiness.com.au/news/probiotics-may-be-used-to-protect-salads-from-salmonella
- http://www.ausfoodnews.com.au/2016/11/16/adding-probiotics-to-salad-may-help-fight-salmonella-poisoning.html
- https://www.foodsafety.com.au/news/healthy-bacteria-to-be-introduced-to-australian-salad-greens

5. Meetings with a large fresh cut salad producer based in Brisbane on 16th December, 2016 and 17th March, 2017.

6. *A/Prof Mark Turner presented **"*Using probiotics to protect (and value add) bagged salads*"** at the FIAQ 13th Annual Food Safety Conference, Brisbane, 1st August, 2017.

7. *A/Prof Mark Turner presented **"Salmonella *growth and control on leafy greens*"** at the Fresh Produce Safety Centre Conference, Sydney, 9th August, 2017

8. A/Prof Mark Turner presented ***"Value added salads using probiotics and biocontrol agents of* Salmonella *on leafy greens"*** at the meeting with the industry partner on 24th November, 2017.

9. *Dr Van Ho presented ***"Application of lactic acid bacteria in food safety and quality"*** at the Centre for Food Safety & Innovation Annual Forum, Melbourne, 7th February, 2018.

10. *A/Prof Mark Turner presented ***"Harnessing environmental biocontrol lactic acid***

bacteria for fresh produce safety" at the FoodMicro 2018 Conference, Berlin, September 2018.

11. *A/Prof Mark Turner and Dr Van Ho had a meeting with another large fresh cut salad producer on 29th March, 2019. A/Prof Mark Turner presented ***"Value added salads using probiotics and biocontrol agents of* Salmonella *on leafy greens"*** at this meeting.

-* The slides for these presentations have been provided to Hort Innovation as pdf files.

Outcomes

1. Development of natural biocontrol bacteria (termed *Probisafe*) for industry application to control pathogen growth on vegetables

1.1. Laboratory trials

1.1.1. Confirmation of anti-*Salmonella* activities of biocontrol lactic acid bacteria

Isolates of lactic acid bacteria found to have anti-*Salmonella* activity in the previous project were re-tested against *S*. Typhimurium ATCC 14028 using an agar spot assay. Twenty five isolates with strong inhibition zones and one isolate with no inhibition zone were selected for further investigation. These isolates were confirmed to be *Weissella*, *Leuconostoc* and *Lactococcus* strains by sequencing the 16S rRNA gene (data not shown). The inhibition zones produced by LAB isolates against *S*. Typhimurium are illustrated in Fig. 1.

Fig. 1 Zone of inhibition produced by LAB isolates against *Salmonella* Typhimurium ATCC 14028

1.1.2. Development of double layer agar for enumeration of injured *S*. Typhimurium cells

A few trials in the effects of biocontrol strains on the growth of *S*. Typhimurium in cut lettuce revealed that the pathogen was occasionally not detected on XLT4 agar, a selective medium, on day 7 of storage (Fig. 2). These results raised the questions that whether *S*. Typhimurium cells were killed by some biocontrol strains or they were injured and unable to recover on XLT4 agar.

When exposed to sublethal environmental stresses, bacterial cells undergo metabolic injury and are unable to form colonies on selective agars because the selective agents can inhibit the repair of injured cells (McCleery and Rowe, 1995). For accurate enumeration of *Salmonella* cells, pre-enrichment media such as lactose broth and buffered peptone water (BPW) are commonly used for increasing the recovery of injured cells (Liao and Fett, 2005). Nonselective media including tryptic soy agar, plate count agar and BHI agar have been used to recover injured *Salmonella* cells (Gurtler, 2009).

In the current study, the resuscitation in BPW and the plating on double layer agar were used to compare the recovery of *S.* Typhimurium treated with LAB isolates. Figure 3 shows the counts of *S.* Typhimurium on cut iceberg lettuce treated with two LAB strains 38 and

752 using the resuscitation method and the double layer agar. The resuscitation of treated lettuce in BPW before plating *Salmonella* on selective XLT4 agar gave approximately 10-100 fold higher counts than the normal plating method without resuscitation. It was noticed that *Salmonella* in lettuce inoculated with the strain 752 was undetectable on XLT4 agar after 7 days of storage. However, their counts (10^3 CFU/g) obtained from direct plating of non-resuscitated *Salmonella* cells on the newly developed media containing XLT4 and BHI agar were similar to those from the resuscitation method. The disadvantage of the resuscitation method is that the uninjured cells can multiply during the recovery time of the injured cells. Therefore, this method would not be appropriate for accurate enumeration of *S.* Typhimurium on treated lettuce. In the double layer agar, the diffusion of nonselective BHI agar to the top layer would allow injured *Salmonella* to be resuscitated and a typical reaction (black colonies) would produce due to the selective agents from XLT4 agar. The use of the double layer agar shortens the time for enumeration of *Salmonella* as the resuscitation step is not required and the agar plates are incubated overnight instead of 48 h in the case of XLT4 agar.

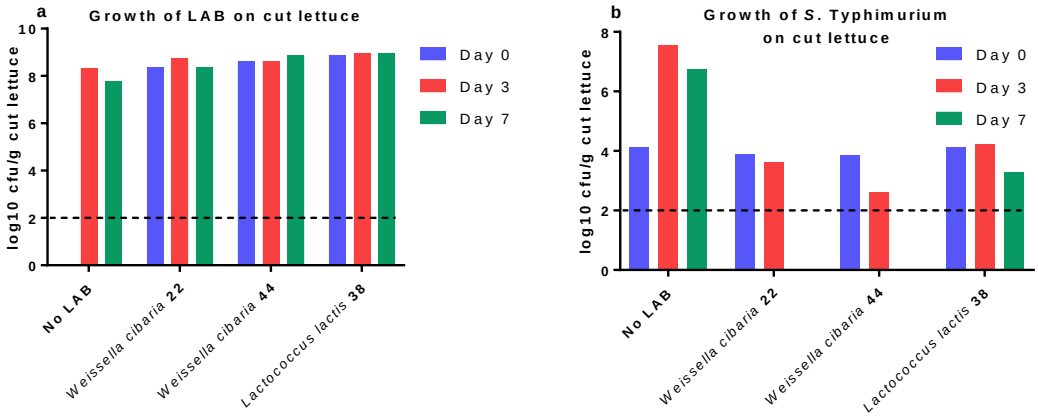

Fig. 2 Effect of biocontrol bacteria (a) on growth of *S.* Typhimurium on cut iceberg lettuce at 8 ˚C

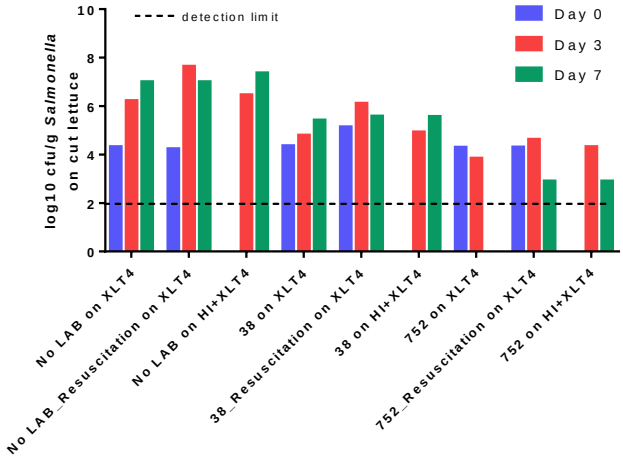

Fig. 3 Growth of *S.* Typhimurium on cut iceberg lettuce before and after resuscitation

1.1.3. Attachment of *Probisafe* isolates on cut iceberg lettuce under different treatment times

The effect of contact time on the attachment of two LAB isolates, *L. lactis* 38 and *W.*

cibaria 752 on cut lettuce was investigated by adding bacterial cell suspensions onto lettuce samples and left them for 1, 5, 15 and 30 min. The populations of LAB in lettuce over 7 days of storage are shown in Fig. 4. The inoculation resulted in initial LAB counts of 10^8-10^9 CFU/g which remained relatively unchanged over 7 days of storage for all 4 treatment times. These results suggest that LAB isolates attached very well in lettuce even under a short contact time of 1 min. To make the experiment more realistic and feasible, the treatment time of 5 min was chosen to use in further studies.

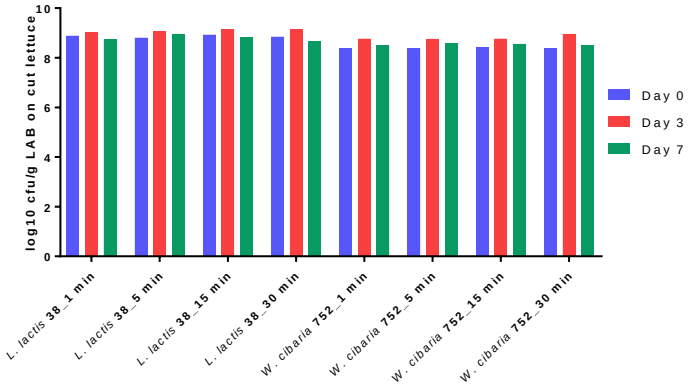

Fig. 4 Growth of LAB isolates under different treatment times

1.1.4. Effects of different initial concentrations of *Probisafe* strains on lettuce quality

Three different initial concentrations (10^7, 10^8 and 10^9 CFU/g shredded lettuce) of two selected LAB strains (*L. mesenteroides* 109 and *W. cibaria* 752) and constant soaking time (5 min) and storage temperature (8 °C) were used to evaluate their effects on iceberg lettuce quality. The growth of LAB in iceberg lettuce over 7 day storage is shown in Fig. 5. Expected initial concentrations of two LAB strains were achieved and the bacteria attached very well in lettuce under a contact time of 5 min. The initial populations of LAB strains generally remained at similar levels over 7 days of storage, except a slight increase of about 0.5 log in the initial inoculum of 10^7 CFU/g. Initial levels of LAB strains affected the pH of lettuce samples and, consequently, on lettuce appearance. Lettuce samples inoculated with initial bacterial levels of 10^9 CFU/g had lower final pH values of 3.9-4.2 and a softer texture (data not shown) compared to other lower initial bacterial concentrations. Increasing the initial bacterial concentration resulted in the reduction of lettuce's pH (data not shown) due to the higher production of organic acids by LAB strains. LAB strains could also produce pectinolytic enzymes which cause vegetable tissues to soften and these enzymes are more active at lower pH (Peréz-Díaz et al., 2013). To increase the inhibition of *Salmonella* by LAB strains without compromising sensory properties of shredded lettuce, initial bacterial concentrations of 10^8 CFU/g were chosen for further studies.

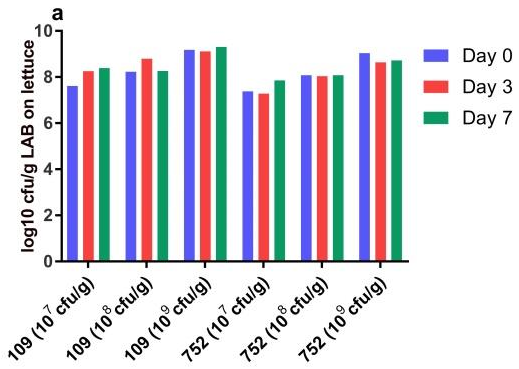

Fig. 5 Growth of LAB strains at different initial counts in lettuce

1.1.5. Inhibition of *S*. Typhimurium growth by *Probisafe* strains on ready-to-eat whole and cut leafy vegetables

Several trials were carried out to investigate the effects of selected biocontrol strains on the growth of *S.* Typhimurium in whole and cut leafy vegetables stored at 8 °C for 7 days. Biocontrol strains were inoculated onto vegetables at initial populations of 10^8-10^9 CFU/g which remained at these levels throughout 7 days of storage (data not shown). The inoculation of *S.* Typhimurium resulted in initial counts of 10^3-10^5 CFU/g on vegetables. In the samples without inoculated LAB isolates, *Salmonella* grew to maximum populations of 10^7-10^8 CFU/g in the cut lettuce and to 10^5-10^7 CFU/g in other vegetables (Fig. 6). Cutting of iceberg lettuce enable the release of nutrients which may promote faster growth of bacteria. The cutting also exposes internal tissues allowing the transfer of bacteria from outer surface. Cut vegetables may be subject to higher risks of *Salmonella* contamination compared to whole produce (Abadias et al., 2008; Harris et al., 2003).

Biocontrol strains including 22, 752, 733 and 838 showed no inhibition of *Salmonella* growth in baby spinach and rocket (Fig. 6a, 6c). However, the growth of *S.* Typhimurium was inhibited by about 1 log CFU/g on shredded cabbage and carrot inoculated with the strain 752 (Fig. 6b) and up to more than 4 log CFU/g on lettuce inoculated with the strains 22 and 44 (Fig. 6d). *L. lactis* 38 was a negative control which only showed minor inhibitory activity.

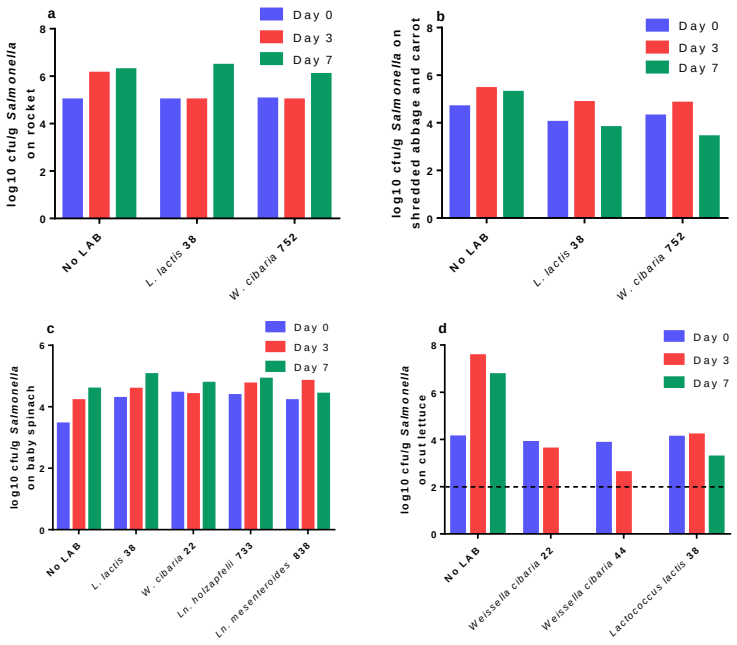

Fig. 6 Effects of biocontrol strains on growth of *S.* Typhimurium in baby rocket (a), shredded cabbage and carrot (b), baby spinach (c) and cut iceberg lettuce (d)

1.1.6. Preparation of cut iceberg lettuce using 'spin' and 'no spin' methods

As shown by the results in Table 4, there was little difference in moisture content and water activity among the samples. Therefore our treatment methods, regardless of whether spinning was involved, produced lettuce with moisture content and water activity that are similar to commercial pre-cut lettuce.

In addition, we performed two other experiments in which we monitored the counts of two LAB strains in lettuce (*L. mesenteroides* 109 and *W. cibaria* 752) that were spun and not spun over 7 days. Two experiments were performed: one stored at 8 °C and another stored at 4 °C. There was little difference in the counts between the spun and not spun samples, except for the no LAB control stored at 4 °C, which had higher counts in the sample that was not spun (Fig. 7). However, the counts were low and these were just the background LAB in the lettuce.

Table 4 Moisture content and water activity of lettuce that was spun and not spun.

	Moisture content (%)*	Water activity
Commercial	96.7	0.991
Spin (95 g)	96.0	0.986
No spin (95 g)	96.1	0.990
No spin (10 g, tube)	96.2	0.987
No spin (10 g, bag)	96.5	0.986

*Each result is a mean of duplicate samples, rounded off to 3 significant figures.

Since spinning made little difference to the moisture content, water activity and LAB counts, in the interest of simplicity and minimising aerosol containing *Salmonella*, we concluded that we would not use a salad spinner to remove excess cell suspension from the lettuce. Our method of decanting and pipetting was a suitable alternative that resulted in lettuce with similar moisture content and water activity as commercial pre-

cut lettuce.

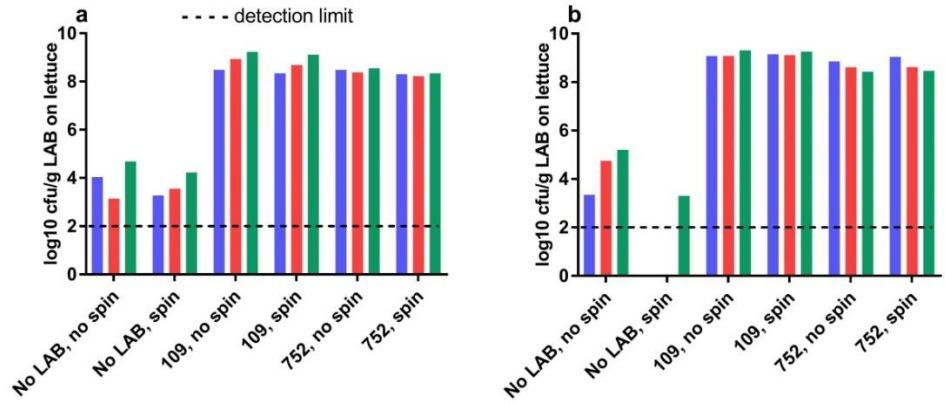

Fig. 7 LAB counts in lettuce that were spun and not spun after soaking in bacterial cell suspension over storage for 7 days. Two experiments were done: a, storage at 8 °C; b, storage at 4 °C. Control with no LAB were soaked in normal saline (0.85% NaCl), the same diluent used for preparing the LAB cell suspensions.

1.1.7. Application of *Salmonella* in cut lettuce by spotting vs soaking

For both LAB and *Salmonella*, there was no growth or even slight decrease in cell numbers in the spotting samples (Fig. 8). In particular, there was a 0.744 log (or 5.5 times) decrease of *Salmonella* on day 7 compared to day 0 in the no LAB control. In contrast, the soaking samples showed growth over 7 days. On day 7, the *Salmonella* counts were 1.64, 1.23 and 1 log (or 43, 17 and 10 times) higher compared to day 0 in the no LAB, 109 and 752 samples, respectively.

Growth of *Salmonella* in the no LAB sample is important as it allows a clear demonstration of inhibition, if any, by the candidate biocontrol strains. The lack of *Salmonella* growth in samples using the spotting method makes it an inappropriate method, even though it is simpler and safer than soaking. One possible explanation for the poor growth of *Salmonella* in the spotting method is that the cells were localised in a small area, thus limiting their nutrient uptake. Therefore it was concluded that spotting is not suitable for our purposes and the soaking method would be used in subsequent experiments.

It is noteworthy that the 109 and 752 strain did not show much inhibition of *Salmonella* here (Fig. 8b). This is most likely due to lower initial counts of LAB (10^6–10^7 CFU/g, as opposed to the usual level of 10^8 CFU/g).

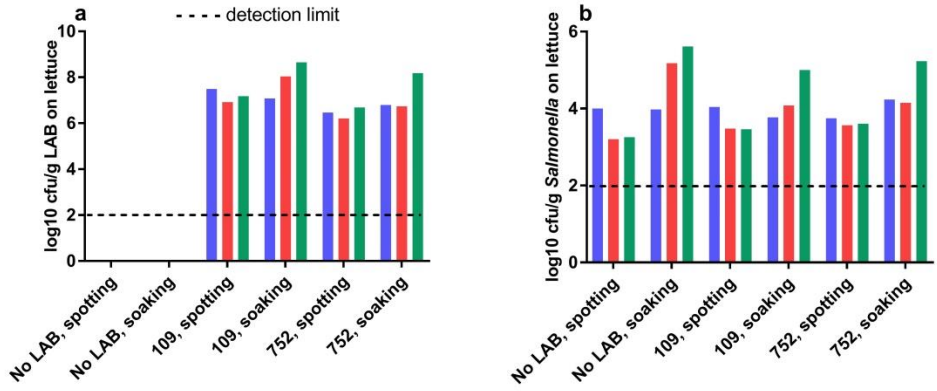

Fig. 8 Comparing the growth of LAB (a) and *Salmonella* (b) in lettuce with *Salmonella* applied by spotting or soaking over 7 day storage at 8 °C.

1.1.8. Inhibition of *Salmonella* in iceberg lettuce by *Probisafe* strains at different storage temperatures

Twenty eight LAB strains showed promising inhibition against *S.* Typhimurium in the agar overlay assay were tested in shredded iceberg lettuce stored at two different temperatures. The growth of LAB isolates and *S*. Typhimurium in lettuce at 4 and 12 °C is shown in Fig. 9, 10, 11 and 12. Most of biocontrol strains, except strains 498 and 824, were inoculated into lettuce at initial populations of 10^8-10^9 CFU/g which remained at similar levels after 7 day storage at 4 °C (Fig 9) whereas they increased approximately 0.5-1 log CFU/g when stored at 12 °C (Fig. 11). Initial counts of strains 498 and 824 in lettuce were 10^7-10^8 CFU/g which increased more than 1 log at both storage temperatures. *S*. Typhimurium was inoculated into lettuce at initial concentrations of about 10^3-10^4 CFU/g which decreased by 0.5-1 log CFU/g in all samples over 7 day storage at 4 °C (Fig. 10).

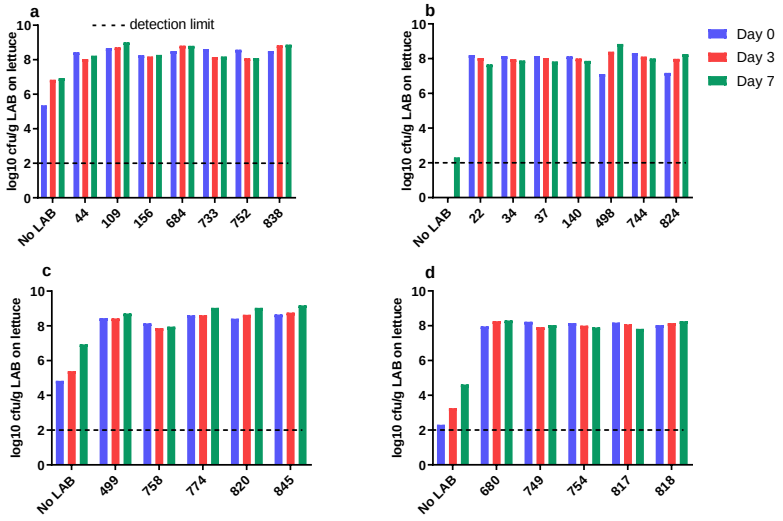

Fig. 9 Growth of LAB in shredded iceberg lettuce stored at 4 °C

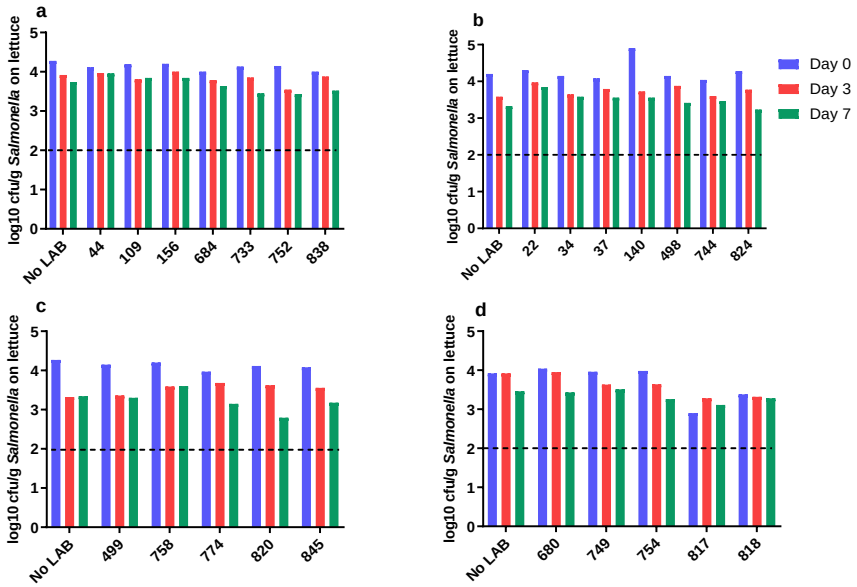

Fig. 10 Growth of *S*. Typhimurium in shredded iceberg lettuce stored at 4 °C

When lettuce was stored at 12 °C, however, *Salmonella* counts increased about 3 log to 10^6-10^7 CFU/g in the control samples without inoculated LAB strains. The growth of *S.* Typhimurium in lettuce held at 12 °C was inhibited in all samples inoculated with biocontrol strains compared to the control. The inhibition of *Salmonella* by LAB isolates was strain dependent. Out of 28 LAB isolates tested, 18 strains (22, 34, 37, 44, 140, 156, 493, 498, 538, 680, 749, 754, 757, 758, 786, 817, 818 and 824) inhibited *Salmonella* growth by less than 1 log CFU/g and 5 strains (499, 684, 752, 744 and 838) showed an inhibition of 1.2-1.7 log CFU/g (Fig. 12). The strongest inhibition against *S.* Typhimurium ATCC 14028 in lettuce stored at 12 °C was found in the samples inoculated with the 109, 733, 774, 820 and 845 strains which showed a decrease of *Salmonella* growth up to 3.7 - 5.9 log CFU/g compared to the control (Fig. 12).

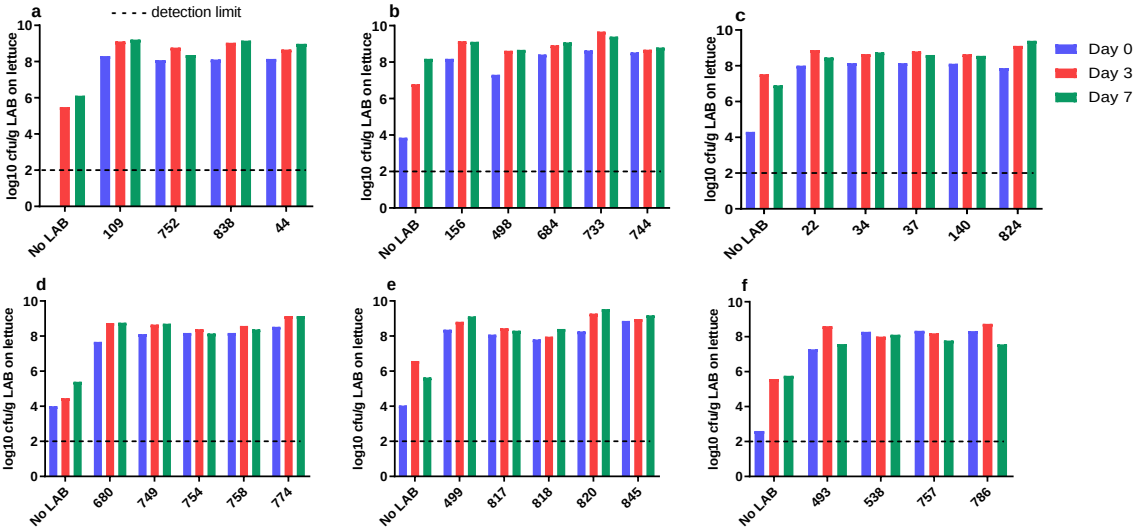

Fig. 11 Growth of LAB in shredded iceberg lettuce stored at 12 °C

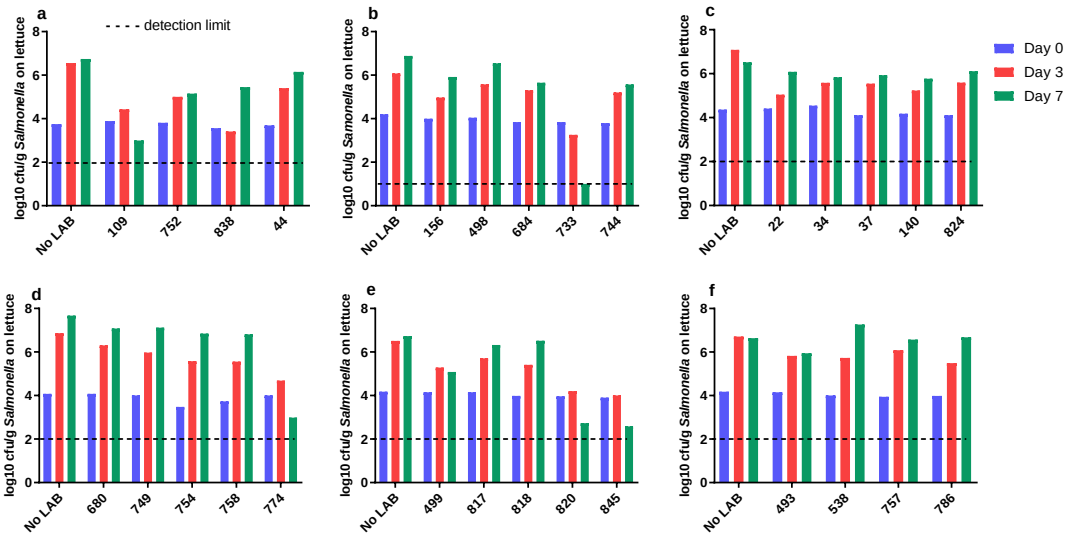

Fig. 12 Growth of *S*. Typhimurium in shredded iceberg lettuce stored at 12 °C

The changes in the appearance of shredded iceberg lettuce treated with and without biocontrol strains, packaged in MAP conditions and stored at 4 and 12 °C are shown in Fig. 13 and 14. All lettuce samples, except samples inoculated with the 733 and 820 strains, maintained a good visual quality during 7 day storage at 4 °C (selected samples shown in Fig. 13). The appearance of shredded lettuce stored at 12 °C remained unchanged up to 3-4 days, however, after that the lettuce lost crispness and became soft towards the end of the storage period (Fig. 14). Lettuce samples inoculated with the 733 and 820 strains did not maintained a good visual quality after 2-3 day storage at both temperatures of 4 and 12 °C.

Fig. 13 Appearance of lettuce after 7 days stored at 4 °C

Fig. 14 Appearance of lettuce after 7 days stored at 12 °C

The results revealed that storage of shredded lettuce at 4 °C allowed *S*. Typhimurium to survive, but suppressed its proliferation whereas storage at 12 °C stimulated the growth of this pathogen. Our results were in agreement with the study by Sant'Ana et al. (2012) and Tian et al. (2012). Although the appearance of cut lettuce samples stored at 12 °C declined significantly by the end of storage period (Fig. 14), their visual quality was still fully acceptable until days 3-4 (data not shown) when *Salmonella* growth reached a high levels of 10^5-10^7 CFU/g (Fig. 12). These findings emphasise the importance of keeping fresh-cut vegetables at cold temperature of 5 °C or lower for maintaining fresh appearance and reducing food safety risks (Oliveira et al., 2010). The exposure of these products to abusive temperatures during storage can facilitate rapid quality deterioration due to the proliferation of spoilage bacteria and may result in the growth of pathogens to potentially dangerous levels (Jacxsens et al., 2002; Little and Gillespie, 2008; Smyth et al., 1998).

Compared to agar overlay assays, the effectiveness of LAB isolates against *S*. Typhimurium varied in lettuce systems. The differences in the anti-*Salmonella* activity of the biocontrol strains may be attributed to their abilities in production of antimicrobial compounds and the interference of naturally occurring microbiota. Antimicrobial activities of LAB result from several mechanisms of action and are mainly associated with the production of bacteriocins, organic acids, hydrogen peroxide and diacetyl (Cleveland et al., 2001; Trias, Badosa, et al., 2008) and their competition for physical space and nutrients (Galvez et al., 2010; Liao and Fett, 2001). Out of 28 LAB isolates tested in our study, the highest *Salmonella* reduction of 3.7 to 5.9 log CFU/g occurred in lettuce samples inoculated with the 109, 733, 774, 820 and 845 strains and held at 12 °C (Fig. 12). Although the 733 and 820 strains remarkably inhibited *Salmonella* growth in fresh-cut lettuce, its adverse effects on visual quality of lettuce at both storage temperatures limited the application of this strain in ready-to-eat leafy vegetables. The 109, 774 and 845 strains appeared to be a good potential as a biocontrol agent in fresh produce. Cut lettuce samples inoculated with these 3 strains maintained a good quality during storage period at the recommended temperature of 4 °C which suppressed *Salmonella* growth and they had a decline in visual quality after 3 day storage at the abusive temperature of 12 °C which supported *Salmonella* growing to levels likely to represent a food safety problem (Fig. 12a, 12d and 12e). Therefore, the 109, 774 and 845 strains have the potential to apply in washing solutions of fresh-cut lettuce for

suppressing *Salmonella* growth to dangerous populations when the products are exposed to inappropriate storage temperatures. In addition, the abusive temperatures which favoured the proliferation of *Salmonella* also facilitated the deterioration in visual quality of lettuce inoculated with the 109, 774 and 845 strains. Thus, these biocontrol strains can be used as an indicator in fresh-cut lettuce for the presence of *S.* Typhimurium at potentially dangerous levels.

All *Salmonella* serotypes are considered potential human pathogens. It is required that *Salmonella* is absent in 25 g of food sample. The presence of *Salmonella* in 25 g of a sample is considered as potentially hazardous and is unacceptable for consumption, requiring product disposal or recall (Food Standards Australia New Zealand, 2016). Therefore the levels of *Salmonella* we have applied to the vegetables are unsafe, however are necessary to allow detection/quantification in our trials. In the industry, there could be rare sporadic low level of contamination of *Salmonella* (e.g. from wild birds or rodent faeces on the vegetables on farm), which during storage could grow and increase in numbers to potentially dangerous levels. *Probisafe* strains would have the application in controlling this growth to maintain low or undetectable levels of *Salmonella* in the event of a rare contamination.

1.2. Industry trial

1.2.1. Development of food grade media to grow *Probisafe* bacterial strains

After 24 hours of incubation with the three most promising *Probisafe* strains *Leuconostoc mesenteroides* (109, 774 and 845) levels of viable bacteria exceeded 10^9 CFU/ml (Fig . 15). Growth in normal non-food grade media (MRS broth) achieved numbers of between 2-5x10^9 CFU/ml. Therefore these food grade media produce around 2 to 5-fold lower numbers, but still very good – our goal was to achieve > 10^9 CFU/ml using food grade media which was achieved. This will allow for some loss of viability during spray drying and storage. Spray drying the cultures will provide a highly concentrated, stable and easy-to-apply source of *Probisafe* for the future applications. The levels of the three most promising *Probisafe* strains were highest when cultured in formula D and therefore this formula was selected to grow *Probisafe* strains for industry trials.

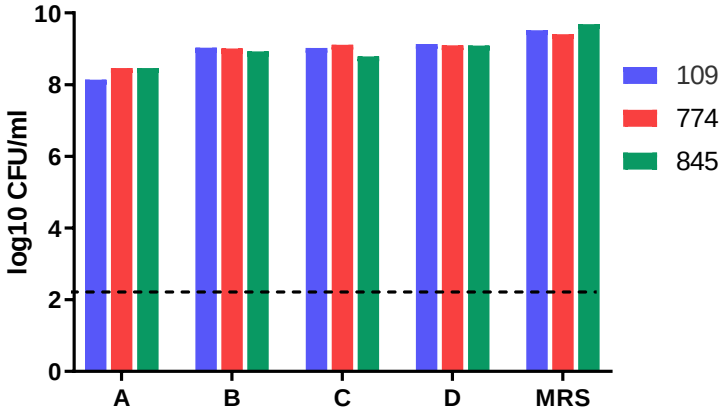

Fig. 15 *Probisafe* strain numbers following incubation for 24 hours in the food grade growth media formulations shown in Table 2.

1.2.2. Growth of *Probisafe* strains in shredded iceberg lettuce in the industry trial

The growth of the three most promising *Probisafe* bacteria in shredded iceberg lettuce from the industry trial is shown in Fig. 16. Those strains were inoculated into lettuce at initial populations of around 10^8 CFU/g which increased to 10^9 CFU/g after 7 day storage at 12 °C. The growth of *Probisafe* bacteria in lettuce and the changes in lettuce appearance in the industry trial were similar to those of laboratory trials (see Section 1.1.8).

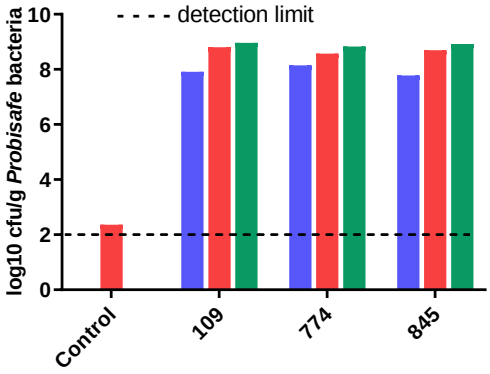

Fig. 16 Growth of *Probisafe* bacteria in shredded iceberg lettuce

1.3. Safety evaluation and whole genome sequences of LAB strains

- FSANZ dossier provided in Appendix C, but will contain specific strain information described below.

1.3.1. Biogenic amines

Biogenic amines are formed by the microbial decarboxylation of amino acids and transamination of aldehydes or ketones. They are detected in a variety of foods including meat, dairy, fruits, vegetables, seafood, nuts and fermented products (Tenbrink et al., 1990). The most common biogenic amines found in foods such as seafood and meat products are histamine, putrescine, cadaverine, tyramine, 2-phenylethylamine and tryptamine (Biji et al., 2016; Demeyer et al., 2000; HernandezJover et al., 1996). Consumption of excessive levels of biogenic amines can pose a health risk as they may cause toxicological symptoms and can be potential carcinogens (Shalaby, 1996). Therefore, the detection of biogenic amines in foods is of great interest.

Lettuce samples were prepared with three different species of candidate biocontrol LAB strains (*L. mesenteroides* 109, *W. confusa* 744 and *W. cibaria* 752) and packaged under MAP. After storage at 8 °C for 5 days, the samples were sent to Symbio Laboratories (Brisbane, QLD) for detection of four types of biogenic amines. The initial LAB counts in the samples were 10^8 CFU/g and these levels were maintained till day 7 of storage.

The concentrations of the amines are shown in Table 5 and the certificate of analysis was attached in the Appendix B. β-phenylethylamine and histamine were below the detection limit of 2 mg/kg in all the samples, while putrescine and cadaverine was found, except the 109 sample which had undetectable levels of cadaverine.

There are no FSANZ limits for these amines in vegetables. According to the Australian New Zealand Food Standards Code – Standard 2.2.3, the only limit that applies to these amines is for histamine in fish or fish products, which cannot exceed 200 mg/kg. This requirement is consistent with the values set by the European Commission Regulation

(Collins et al., 2011). Small amounts of putrescine occur naturally in vegetables, and cadaverine has also been reported to be present (Coelho et al., 2005). Studies have found average putrescine levels in beer and wine of 3 and 5 mg/L, in cheese at ~30mg/kg, fermented sausages at 80mg/kg and fermented vegetables at 250mg/kg (EFSA, 2011). Levels of cadaverine has also been reported in cheese at 80mg/kg and fermented vegetables at 30mg/kg (EFSA, 2011). Varying levels of putrescine in iceberg lettuce have been reported in independent studies, ranging from 5.3 to 16 mg/kg (Pinto and Ferreira, 2015; Simon-Sarkadi and Holzapfel, 1994). Cadaverine was undetected in iceberg lettuce in one study (Simon-Sarkadi and Holzapfel, 1994), while another study on American lettuce found 1.7 mg/kg of cadaverine (Coelho et al., 2005). The current results are comparable to these values found in the literature. **Therefore we conclude that the addition of 10^8 CFU/g of our LAB strains and storing for at least 5 days at 8 °C do not result in elevated biogenic amine levels that might endanger health.**

Table 5 Concentration of biogenic amines in lettuce samples treated with LAB.

	Concentration of amine (mg/kg)		
	109	744	752
β-phenylethylamine	<2	<2	<2
Putrescine	19	20	18
Cadaverine	<2	5.3	2.3
Histamine	<2	<2	<2

1.3.2. Whole genome sequences of LAB strains

1.3.2.1. Virulence

Whole genome sequencing was performed on 12 biocontrol LAB strains belonging to the genera *Weissella* and *Leuconostoc* (Table 3). In order to determine whether the sequenced LAB strains carry genes that might confer virulence, amino acid sequences of the virulence factors of *Listeria monocytogenes*, *Staphylococcus aureus* and *Streptococcus* listed in the Virulence Factor Database (VFDB) (http://www.mgc.ac.cn/VFs/main.htm, (Chen et al., 2016)) were collected and searched against all the genomes in Geneious using tBLASTn. The virulence genes that were not found in any of the genomes are listed in Table 7 in Appendix A. Overall, the majority of highly specific virulence genes were not found in the LAB genomes. None of the toxin genes of *L. monocytogenes* and *S. aureus* and the superantigens have matches in the LAB genomes. The type VII secretion system genes of *S. aureus* and the majority of toxin genes in *Streptococcus* were also not found.

However, it was found that 10 out of the 12 genes in the *cyl* operon which is necessary for the production of β-hemolysin/cytolysin in Group B *Streptococcus* (GBS) have homologues in the LAB genomes (Table 8). This operon is also required for the production of the GBS pigment granadaene, which is an ornithine rhamnolipid. The proteins CylD, CylG, ApcC, CylZ and CylI are homologues of enzymes involved in bacterial fatty acid biosynthesis, and CylA and CylB are components of an ABC (ATP-binding cassette) transporter. Therefore it is not surprising to find homologs of these genes in most if not all of our strains. However, there has been some controversy on the role of *cylE*. These gene was previously thought to be the structural gene encoding the novel proteinaceous β-hemolysin (Pritzlaff et al., 2001). However, this proposition was

overturned in a recent study that showed β-hemolysin is in fact granadaene, not a protein, and that *cylE* was proposed to function as a N-acyltransferase in the grandanene biosynthetic pathway (Whidbey et al., 2013). Further studies to purify the elusive β-hemolysin are required to conclude whether the hemolysin and the pigment are the same molecule (Rosa-Fraile et al., 2014). *Leuconostoc lactis* 824 stands out among all the sequenced LAB strains to contain homologues of the entire *cyl* gene cluster. It is possible that this gene cluster is only involved in producing fatty acid(s) (no pigment has been noticed in this strain), and further testing is necessary to show toxin production. However, these presence of homologues of the entire *cyl* operon suggests a safety issue in *Leuconostoc lactis* 824 and that this strain is not an ideal candidate for biocontrol purposes and will be excluded from future evaluation.

Also noteworthy is the finding of homologues to internalin genes (*inl*) of *L. monocytogenes* in 8 LAB strains (Table 9). However, these are all partial matches, with a coverage of 53–76% and identity of 26–34% in the predicted protein. Moreover, non-pathogenic *Listeria*, *Lactobacillus* spp. and the probiotic strain *Lactobacillus plantarum* WCFS1 also encode internalins (Leisner et al., 2012). Therefore the internalin-like proteins in the LAB under study are not necessarily associated with virulence.

Some virulence factors are non-specific, i.e., they are housekeeping genes or genes that are part of the normal physiology of bacteria. Matches to such genes include metal transporters, sortases, D-alanine--poly(phosphoribitol) ligase, bifunctional acetaldehyde-CoA/alcohol dehydrogenase, oligopeptide-binding protein, serine protease and type I glyceraldehyde-3-phosphate dehydrogenase. The presence of these homologues in the LAB strains is normal and poses no safety concerns. There are also matches to some genes involved in capsule biosynthesis and adhesion, including fibronectin-binding proteins, choline-binding proteins and collagen-binding protein. The probiotic strain *L. plantarum* WCFS1 carries genes predicted to encode fibronectin-binding protein and various capsule biosynthesis proteins (Leisner et al., 2012). Further evaluation will provide a clearer picture with regard to the significance of such matches.

Due to the large scope of virulence analysis, it will be continued and further relevant results will be reported. **Currently we have no reason to suspect that *Probisafe* strains (excluding strain 824) have potential safety issues with regards to virulence genes, since these genes are also present in other regularly consumed and safe probiotic and fermentation bacteria.**

1.3.2.2. Antibiotic resistance

Many LAB naturally contain antibiotic resistance genes. Antibiotic resistance in itself is not a safety issue. It is only when there is a risk of transfer of resistance genes to pathogens that such LAB becomes a safety concern. To definitively evaluate the possibility of antibiotic resistance transfer, mating studies between bacteria strains such as conjugation are needed. However, the genetic context of the antibiotic resistance gene in question can provide good clues regarding the possibility of transfer. The presence of plasmid-associated genes such as *rep* and mobile elements such as insertion sequences, recombinase/transposase in close proximity with the antibiotic resistance gene suggests a high possibility of transfer to other strains (Florez et al., 2016; Gueimonde et al., 2013).

A preliminary survey was undertaken by collating the antibiotic resistance genes

annotated in RAST and is presented in Table 10. Three probiotic *Lactobacillus* strains were also included for comparison. Three main classes of antibiotic resistance genes were found in the LAB genomes: resistance to fluoroquinolones, β-lactamases and multidrug resistance pumps. Only *Weissella soli* 498 and the three *Lactococcus lactis* strains were not found to carry genes encoding β-lactamase. All the other strains carried genes encoding at least one class of β-lactamase. The most common class of β-lactamase gene found in these strains is *BLc, which encodes β-lactamase class C and other penicillin-binding proteins. All strains carry genes encoding at least one type of multidrug resistance efflux pump. The most common type is MFS (multidrug-efflux transporter, major facilitator superfamily (MFS)), followed by the MATE family MDR pump. It should be noted that the three commercially used and common probiotic *Lactobacillus* strains also carry some of these genes, so their presence in our strains is not necessarily an immediate cause for concern.

The genome sequences of our candidate biocontrol LAB strains were uploaded into CARD (Comprehensive Antibiotic Resistance Database) for a more comprehensive search against curated antibiotic resistance genes (Jia et al., 2017). Specifically, the Resistance Gene Identifier (RGI) tool of CARD was used. RGI has three algorithms for analysing the input sequences: perfect, strict and loose. The default setting in RGI is to return hits with the perfect and strict algorithm and we found no hits with this setting. In order not to miss out on the detection of antibiotic resistance genes, we expanded the search by using the loose algorithm, which returned more than 120 hits for each strain.

However, upon examination of the hits obtained with the loose algorithm, it was found that they were highly non-specific, including genes that encode for ABC transporters, transcription regulators and translation elongation factors. The loose algorithm is described by the RGI to work "outside of the detection model cut-offs to provide detection of new, emergent threats and more distant homologs of AMR genes, but will also catalog homologous sequences and spurious partial hits that may not have a role in antibiotic resistance" (https://card.mcmaster.ca/analyze/rgi). By using the loose algorithm, we ended up with the opposite problem in which we had too much noise in our results, thus this algorithm seems to be unsuitable for our purposes.

In order to verify that using the default perfect and strict algorithm of RGI would indeed identify antibiotic resistance genes and the lack of results from our strains was not simply misdetection, we input various positive control sequences known to be resistant to at least one antibiotic into RGI (Table 11). These include complete genome sequences and draft genome assemblies of chromosomes and plasmids from multi-drug resistant pathogens and non-pathogenic lactic acid bacteria. With the exception of the *tetL* gene from the pLS55 plasmid of *Lactobacillus sakei* Rits 9, the antibiotic resistance genes of all other tested sequences were identified using the perfect and strict algorithm. They span a wide spectrum of drug classes. Therefore, we conclude that we did not miss out on antibiotic resistance genes in our LAB strains by using the perfect and strict algorithm.

We also examined the antibiotic resistance genes identified by RAST that were not detected by the strict algorithm of CARD RGI. None of them are in close proximity to genes that encode mobile element proteins or appear to be part of plasmids, thus they are not likely to be transferred to other bacteria.

To sum up, based on genome sequence information, there does not seem to be a high risk of transfer of antibiotic resistance genes from the LAB strains studied to other

bacteria.

2. Evaluation of commercial probiotic delivery using vegetables

2.1. Laboratory trials

2.1. 1. Survival of commercial probiotics in baby spinach

Currently, most probiotic foods available in the market are dairy-based products which cannot be consumed by individuals with lactose intolerance, milk protein allergy and cholesterol-restricted diets. The consumption of dairy probiotic products is also limited by an increase in vegetarian/vegan consumers (Granato et al., 2010; Ranadheera et al., 2010). Therefore, the development of non-dairy probiotic products has a promising future. Vegetables offer healthy alternatives for delivering probiotics due to their large distribution and nutritional benefits.

The day 7 counts of all probiotic strains were reduced compared to day 0, but to varying degrees (Fig. 17). Strains B-1 and B-2 had the worst survival at day 7, decreasing by 2 and 4 orders of magnitude respectively compared to the initial counts. By comparison, the decrease of the other strains ranged from 0.03 to 1.3 $\log_{10}$. The three strains showing the least reduction at day 7, thus showing the best survival, were B-3, B-7 and B-8, having 0.03-0.13 $\log_{10}$ lower counts than on day 0. The best surviving strain from company A, A-3, was the same species as B-3. B-1 and B-4 belong to the same species, but show very different survival patterns on spinach. This reinforces the well-established finding that strain differences can affect phenotypes significantly.

As required by the European Union, probiotic bacteria should be present at a minimum level of 10^7 CFU/g in food products (Cousin et al., 2012). Several of probiotic strains tested in spinach meet this minimum level and thus their application in commercial bagged salads is very feasible. Some probiotic strains used in this study have received a Generally Recognised As Safe (GRAS) status and tested in many clinical studies and scientific publications. Therefore, a general level health claim may be made on packaged probiotic vegetables under the Australia New Zealand Food Standards Code – Standard 1.2.7 – Nutrition, health and related claims (Federal Register of Legislation, 2017).

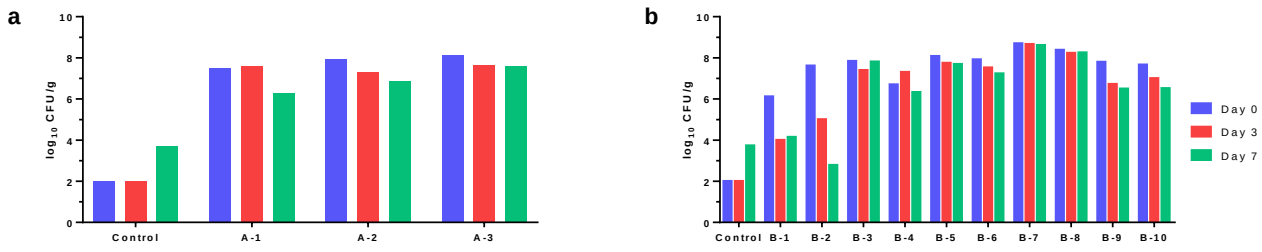

Fig. 17 Survival of commercial probiotics from company A (a) and company B (b) in baby spinach stored at 7 °C.
The control is sample without application of probiotics.

2.1.2. Survival of probiotics in baby spinach in presence of salad dressing

Salads are often consumed with salad dressing, which is usually acidic. It would thus be of interest to determine if the acidity of salad dressing and the washing adversely affects the survival of probiotics in salad. The pH of the Italian dressing was ~ 3, while that of the French and Balsamic dressings were ~ 4. There was little difference in the survival of the six probiotic strains tested in the presence of all three types of salad dressings

compared to the controls (spinach only or in the presence of an equal volume of water) (Fig. 18). Therefore, it can be concluded that the addition of salad dressing during consumption of probiotic-fortified salads would not adversely affect the survival of the probiotics.

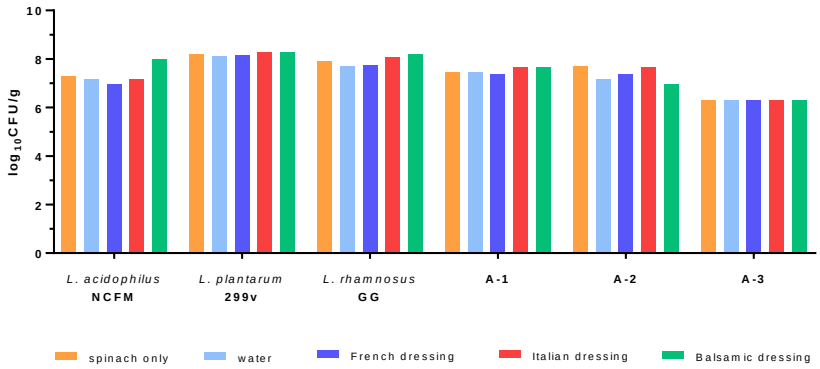

Fig. 18 Survival of probiotic strains in the presence of three types of salad dressing.

2.1.3. Survival of probiotics in simulated gastric and intestinal juices

It is important that the commercial probiotics added to salads survive the harsh conditions of the gastrointestinal tract for them to result in any health benefits to the consumer. Therefore, simulated gastric and intestinal juices were added to baby spinach and the survival of the probiotic strains studied. A control using skimmed milk to replace spinach was tested to determine if dairy components such as proteins or fat offered protection to the strains, resulting in better survival. Overall, the strains showed similar survival patterns in both baby spinach and skimmed milk, with the exception of A-2, which possibly survived slightly better in spinach in the presence of simulated gastric juice (Fig. 19, 20).

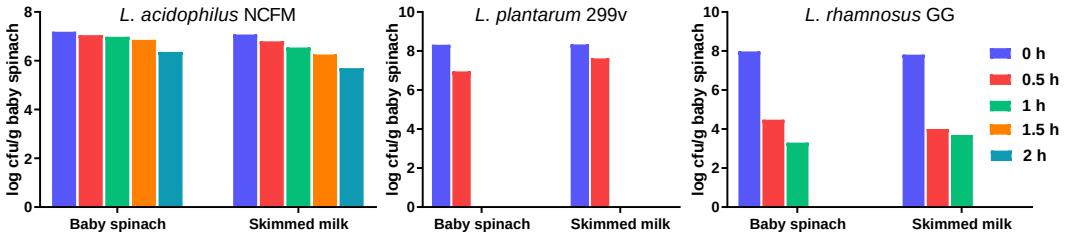

Fig. 19 Survival of commercial probiotic strains in baby spinach and skimmed milk in the presence of simulated gastric juice.

The five probiotic strains tested varied in survival in the presence of simulated gastric juice (HCl added until pH = 2) (Fig. 19). *L. acidophilus* NCFM was the most robust, decreasing by less than 100-fold after 2 h. Strain A-1 was the second best surviving

strain, with 10^2-10^3 CFU/g or CFU/mL still remaining after 2 h. The other three strains were less resistant in the acidic environment. *L. rhamnosus* GG and A-2 showed similar survival patterns. After 1 h, there were ~10^3 CFU/g viable cells, with the exception of A-2 in skimmed milk which was undetectable. *L. plantarum* 299v survived well for 30 min, but it was below detection limit from 60 min onwards. In contrast, all probiotic strains survived well in the presence of simulated intestinal juice (bile salt) (Fig. 20). There was less than 10-fold decrease in viability after 2 h. Therefore, the presence of bile salts did not adversely affect the survival of these probiotic strains tested. The acidic environment in the stomach is a more crucial factor in determining the suitability of these strains to be a good probiotic candidate for salads, however our results suggest that baby spinach as a probiotic carrier provides as much protection from gastric acid as would be expected from a milk-based probiotic food.

Fig. 20 Survival of probiotic strains in baby spinach and skimmed milk in the presence of simulated intestinal juice.

2.1.4. Quality evaluation of baby spinach added commercial probiotics

Two probiotic strains, A and B, which showed good survival in baby spinach were selected for sensory analysis. Before the sensory trials, baby spinach leaves with and without commercial probiotic inoculation were analysed for microbiological safety. As shown in the Appendix B, *Salmonella* spp. and *Listeria monocytogenes* were absent in 25 g of all spinach samples and the counts of *Escherichia coli* and *Staphylococci* were in the acceptable levels for ready-to-eat food products. These results indicated that spinach samples prepared in our study were safe for human consumption. Two probiotic strains tested maintained the populations of 10^7-10^9 CFU/g in spinach leaves during 7 day storage (Fig. 21).

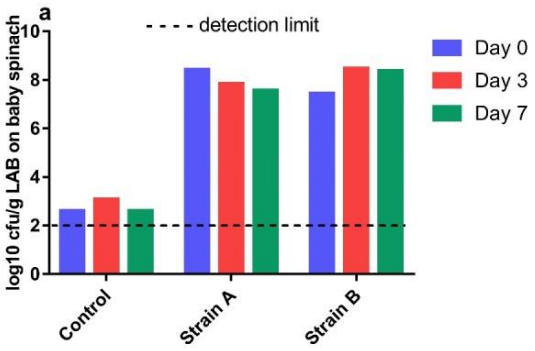

Fig. 21 Survival of commercial probiotic strains A and B in baby spinach stored at 4 °C

All preparations and sensory evaluation were carried out in a certified food product development and food sensory laboratory at the University of Queensland that had registration with the Brisbane City Council for food preparation and consumption. Sensory assessment of probiotic spinach was performed on day 4 of storage. The control and probiotic spinach samples were subjected to sensory evaluation using triangle tests as described in Appendix A (Section 2.3). The triangle test was performed to determine if probiotic containing spinach leaves tasted significantly different from the control spinach without probiotics. The results of the triangle test for sensory evaluation of spinach leaves are presented in Table 6. A total of 40 people participated in the sensory trials and the number of correct judgements (12-13) was less than the minimum number of judgements (19) required to declare for two samples to taste significantly different from one another. Thus, there were no statistically significant differences ($p>0.05$) in the appearance and flavour of spinach leaves inoculated with probiotic strain A or strain B and those of the control samples. Spinach leaves with and without probiotics had similar appearances over 7 day storage at 4 °C as shown in Fig. 22. It may be concluded that the sensory quality of baby spinach was not adversely affected by the addition of two commercial probiotic strains tested.

Table 6 Triangle test for sensory evaluation of baby spinach leaves

Spinach sample	Number of judges	Number of correct judgements	Correct judgements needed for significance * (95% confidence level)
Control vs Probiotic strain A	40	12	19
Control vs Probiotic strain B	40	13	19

** Minimum number of correct judgements needed to declare for two samples to taste significantly different from one another (Adapted from critical value table for triangle test in (Lawless and Heymann, 2010).*

Fig. 22 Appearance of baby spinach after 7 day storage at 4 °C

2.2 Industry trial

The survival of probiotic strains A-2, A-3 and B-8 in baby spinach leaves was shown in

Fig. 23. Probiotic bacteria were added into baby spinach at initial populations of 10^8-10^9 CFU/g which decreased approximately 0.5 log CFU/g after 7 day storage at 4 °C, which remained well above the minimum level of 10^7 CFU/g required by the European Union. These results are in agreement with the laboratory trials. Therefore, the application of probiotics in commercial bagged salads in a non-lab, industrial setting is very feasible.

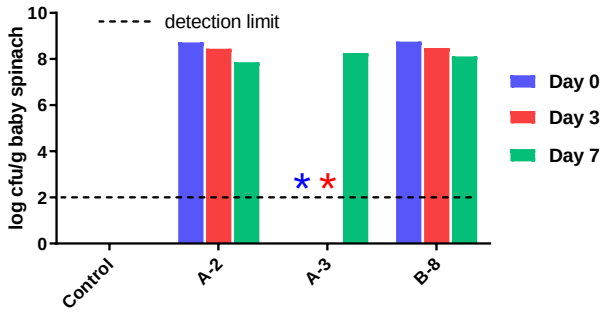

Fig. 23 Survival of commercial probiotic strains in baby spinach during the industry trial

(: data of the A-3 strain on days 0 and 3 were missing due to air leaking from the anaerobic box which was used to incubate MRS agar plates, resulting in no counts for the A-3 strain. The day 7 sample suggests that survival on spinach was excellent with >10^8 CFU/g at the end of the trial)*

Monitoring and evaluation

➢ The project was monitored closely by Uniquest and the project team members at University of Queensland. The Steering Committee was formed with representation from UQ, UniQuest, HIA and a General Manager and a Farm General Manager from a large fresh cut salad producer.

➢ Several meetings between project team members, Uniquest and industry representatives were held at UQ and the site of the industry representatives to gain feedback on project activities.

➢ Project team members met weekly to discuss project methodology and trials.

➢ Milestone reports with achievement-based criteria successfully achieved.

➢ Meetings and discussions with leading probiotic and culture supply company were held at University of Queensland. The probiotic supply company has confirmed that some of their strains have enough documented clinical trial data to make health claims on packaging.

➢ Two large fresh cut salad producers have shown great interest in probiotic vegetables and are keen to start trials of probiotic application in their salad products. We are happy to provide further assistance in the application of this research.

➢ A new category of salad products is anticipated to be released to market in the near future, however this will require further industry action.

Recommendations

➢ Large scale industry trials of probiotic application by industry. The UQ team are happy

to travel to interested companies to assist in technology transfer and provide relevant connections to probiotic suppliers. UQ does not own any IP regarding the commercial probiotic part of the project, so it would be relatively straightforward for a vegetable processor to deal directly with a commercial culture supplier to develop a new product in this area.

➢ Further research on the application methods (soaking, spraying etc.) of adding *Probisafe* or probiotic strains into salad products at commercial scale should be done.

➢ *Probisafe* strains should be produced in a highly concentrated, stable and easy-to-apply source. If the industry is interested in transferring *Probisafe* to their products, then we can discuss with a commercial culture/fermentation company to produce the product in a freeze-dried product.

➢ Effects of *Probisafe* strains on the sensory properties and nutritional quality of salad products during shelf-life should be examined.

➢ Consumer surveys to assess salad consumer's interests in the addition of probiotics should be carried out.

➢ Testing *Probisafe* strains in the laboratory for antibiotic resistance profiles and possibility of transfer of resistance genes to other bacterial strains in conjugation experiments should be made if these strains are to be applied in foods.

➢ Submit dossier (Appendix C) to FSANZ to seek approval for application of commercial probiotics and *Probisafe* strains in foods.

➢ Communication of results to the industry through presentations as horticulture meetings and conferences (e.g. Fresh Produce Safety Centre conference, Hort Connections). Other information releases on the project through the media and communications team at Hort Innovation should be explored.

➢ Publish invited article in *Microbiology Australia* on probiotic vegetables.

Refereed scientific publications

None to report. A PhD student is aligned with this project exploring *Probisafe* activity against *Listeria monocytogenes*, so papers may be published from that work. These will be sent to Hort Innovation for approval before submission to journals.

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IP register provided as an attachment to this report.

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Appendices

Appendix A: Methodology

1. Development of natural biocontrol bacteria (termed *Probisafe*) for industry application to control pathogen growth on vegetables

1.1. Enumeration of biocontrol LAB and *S*. Typhimurium and development of a double layer agar for *Salmonella* enumeration

For enumeration of bacterial populations, vegetables (10-20 g) were aseptically mixed with 0.1 % peptone water at a ratio of 1:10 and homogenised in a stomacher for 1 min to give a uniform suspension of the leaves. One ml of the suspension was serially diluted in 0.1 % peptone water and 0.1 ml samples from each of three consecutive dilutions were spread on plates of MRS agar and XLT4 agar (Oxoid) for LAB isolates and *S.* Typhimurium, respectively. MRS plates were incubated anaerobically at 30 °C and XLT4 plates were incubated at 37 °C under aerobic conditions for 48 h.

To recover injured *Salmonella* cells, a resuscitation method was compared with the use of double layer agar. The resuscitation method was carried out by homogenising 10 g of vegetables with 90 ml of buffered peptone water for 1 min and the mixture was then incubated at room temperature for 4 h before serial dilutions were prepared. The enumeration and incubation of resuscitated *S.* Typhimurium were performed as previously described. The double layer agar was developed by overlaying 10 ml of selective medium (XLT4) onto 10 ml pre-solidified BHI medium. Homogenised samples in 0.1 % peptone water were spread on the overlay agar plates which were incubated aerobically at 37 °C for 24 h.

1.2. Attachment of *Probisafe* isolates on shredded iceberg lettuce under different treatment times

Iceberg lettuce purchased from local supermarkets was removed outer leaves and then washed under tap water to remove dirt prior to being cut into pieces of about 1 cm^2. Ten grams of shredded lettuce were weighed into a sterile bag and treated under UV light for 5 min on each side of the bag prior to being inoculated with 10 mL of bacterial cell suspension, resulting in 10^8 CFU/g of LAB. The bag was then kept on the bench for 1, 5, 15 and 30 min in order to allow bacterial attachment onto lettuce before the suspension was drained off well. Lettuce samples were incubated at 8 ˚C for 7 days.

1.3. Effects of different initial concentrations of *Probisafe* strains on lettuce quality

Shredded iceberg lettuce was prepared as previously described in Section 1.2. A hundred grams of shredded lettuce were weighed into a sterile 1 L container and soaked in 500 mL of bacterial cell suspension for 5 min before the cell suspension was drained off well by decanting and pipetting. The inoculated lettuce was aliquoted into 20 g bags which were applied MAP and stored at 8 ˚C for 7 days. The populations of LAB were counted on days 0, 3 and 7.

1.4. Inhibition of *S*. Typhimurium growth by *Probisafe* strains in ready-to-eat whole and cut leafy vegetables

The bags of leafy vegetables (10 g) were first inoculated with 10 ml of *S.* Typhimurium suspension in 0.85% saline at 10^3-10^4 CFU/ml and gently shaken 30 times to ensure a homogenous distribution of the pathogenic cells. The bags were left in a biosafety cabinet for 15 min to allow the attachment of *S.* Typhimurium onto vegetables before

the cell suspension was drained off. Subsequently, 10 ml of biocontrol strain suspension in 0.85% saline at 10^8 CFU/ml were added and left for 5 min. The bacterial suspension in the bags was then drained off well prior to incubating the samples at 8 ˚C for a period of 7 days. A control sample was prepared in the same manner by adding 10 ml of *S.* Typhimurium suspension and then 10 ml of 0.85% saline without biocontrol strains. The populations of biocontrol strains and *S.* Typhimurium were determined on days 0, 3 and 7 as described in Section 1.1.

1.5. Preparation of shredded iceberg lettuce using spin and no spin methods

Four types of samples (see Table 4) were prepared using spinning and no spinning methods: one spun in the salad spinner and three that were not spun. The reason for testing three different no spin treatments was that our methods have changed over time, and all three methods have been used in our experiments. The lettuce for these samples was cut in our lab from lettuce heads purchased from the supermarket. In the treatments that were designated “no spin” and “spin”, 100 g of shredded lettuce were placed in a box and soaked in 500 mL of saline. This no spin method is the latest method to replace our previous no spin methods in which 10 g of lettuce were placed in a sterile tube ("no spin (tube)") or stomacher bag ("no spin (bag)"), followed by addition of 10 mL of cell suspension and shaking for even distribution. The elimination of the shaking step would reduce injury to the lettuce. The contact time with the saline for all 4 samples was the same as in our experiments in which cells were applied. The moisture content and water activity of these samples were determined alongside commercial pre-cut lettuce.

1.6. Inhibition of *Salmonella* in iceberg lettuce by *Probisafe* strains at different storage temperatures

Two storage temperatures of 4 and 12 ˚C were tested to examine the influence of temperatures on *Salmonella* inhibition in lettuce by *Probisafe* strains. *Salmonella* Typhimurium ATCC 14028 was grown in BHI broth (Oxoid) at 37 ˚C under aerobic conditions. The harvest of LAB and *Salmonella* culture from broth media and the preparation of shredded lettuce were carried out as previously described. Lettuce samples were soaked first in *Salmonella* cell suspension for 10 min and then in LAB cell suspension for 5 min, resulting in initial counts of 10^3-10^4 CFU/g *Salmonella* and 10^8-10^9 CFU/g LAB. The excessive liquid after each soaking was drained off by decanting and pipetting. A control sample without LAB addition was prepared in the same way. After that, aliquots of 20 g lettuce were packed in MAP films which were stored at 4 or 12 ˚C for 7 days. The populations of biocontrol strains and *S.* Typhimurium were determined on days 0, 3 and 7.

1.7. Whole genome sequence

Table 7 Virulence genes that were not found in any of the LAB genomes

Class of virulence factors	*Listeria monocytogenes*	*Staphylococcus aureus*	*Streptococcus*
Adherence	Listeria adhesion protein (*lapB*)	Clumping factor A (*clfA*) Elastin-binding protein (*ebpS*) Fibrinogen-binding protein (*efb*) Poly-beta-1,6-N-acetyl-D-glucosamine synthesis protein (for synthesis of intercellular adhesin) (*icaD*) Extracellular adherence protein/MHC analogous protein (*map*)	Agglutinin receptor Antigen I/II (*spaP*/*pac*, *sspA*, *sspB*) Fibronectin-binding proteins (*sfbI*, *sfbII*, *sfbX*, *fbsA*, *fbsB*, *prtF2*) Collagen binding protein (*cpa*) Serine-rich surface glycoprotein (*hsa*) Cna B-type domain protein of pilus island 1 Major subunit PilB of pilus island 2 Pilus tip adhesin (*rrgA*) and major pilin (*rrgB*) of rlrA islet Cell surface hydrophobicity proteins (*cshA*, *cshB*) Collagen binding protein (*cpa*) Glucan-binding protein (*gbpC)* Protein G-related α 2M-binding protein (*grab*) M protein and M-like proteins (*emm, enn*, *mrp*) R6 surface protein
Enzyme	Zinc metalloproteinase (*mpl*) Phospholipase C (*plcB*) Phosphatidylinositol-specific phospholipase C (*plcA*)	N/A	
Exoenzyme	N/A	Coagulase (*coa*) Hyaluronate lyase (*hysA*) Lipase; glycerol ester hydrolase (*geh*) Lipase 1 (*lip*) Thermonuclease (*nuc*) Staphylokinase (*sak*) Serine protease (*splD*) Serine protease (*splF*) Staphopain A; cysteine protease (*sspB*) Staphostatin B (*sspC*)	EndoS (*endoS*) Hyaluronidase (*hlyB*, *hylA*, *hyl*, *hylP*, *hysA*) Neuramidase A (*nanA*) Streptococcal phospholipase A2 (*slaA*)
Protease	N/A	N/A	Extracellular factor (*epf*) IgG-degrading enzyme (*ideS*/*mac*) IgA1 protease (*iga*) Streptokinase A (*ska*) Cysteine proteinase; pyogenic toxin (*speB*)

			Zinc metalloproteinases (*zmpB*, *zmpC*)
Immune modulator	Listeria nuclear targeted protein A (*lntA*)	N/A	
Host immune evasion	N/A	Capsular polysaccharide biosynthesis protein (two genes) O-antigen ligase family protein Glycosyltransferase WbuAB	Inhibitor of complement-mediated lysis (*sic*) Four capsule biosynthesis genes
Secretion system	N/A	All 8 genes involved in type VII secretion system	N/A
Nucleation promoting factor	Actin assembly inducing protein (*actA*)	N/A	N/A
Surface protein anchoring	Sortase B (*srtB*)	N/A	N/A
Immunoreactive antigen	N/A		α C protein (*bca*) α-like protein (*alp2*) β C protein (*cba*) Rib surface protein Surface immunogenic protein (*sip*)
Superantigen	N/A	N/A	All superantigens listed in VFDG: Mitogenic exotoxin Z (smeZ) Streptococcal pyrogenic exotoxin (*spe*) A, C, G, H, I, J, K, L, M Streptococcal superantigen (*ssa*)
Toxin	Listeriolyin O (*hly*)	All 71 toxin genes listed in VFDB, including genes encoding hemolysins, enterotoxins, exfoliative toxin type A, exotoxins, leukocidins and toxic shock syndrome toxin	CAMP factor (*cfa*/*cfb*) Pneumolysin (*ply*) Suilysin (*sly*) Streptolysin O (*slo*) Streptolysin S (*sagA*) ADP-ribosyltransferase (*spyA*) Two hemolysin/cytolysin genes (*cylX*, *cylJ*)

Table 8 Genes of the hemolysin/cytolysin gene cluster with matches in LAB

	Predicted gene product (Whidbey et al., 2013)	Strains containing matches
cylD	Malonyl-CoA-ACP transacylase	*Leuconostoc lactis* 824
cylG	3-ketoacyl-ACP reductase	All strains
acpC	Acyl carrier protein	*Leuconostoc lactis* 824 *Leuconstoc mesenteroides* 109 *Leuconostoc mesenteroides* 156 *Leuconostoc mesenteroides* 684 *Leuconostoc mesenteroides* 838 *Weissella confusa* 44 *Weissella confusa* 744 *Weissella cibaria* 22 *Weissella cibaria* 752 *Weissella cibaria* 758
cylZ	β-hydroxyacyl-ACP dehydratase	All strains
cylA	ABC transporter ATP-binding protein	All strains
cylB	ABC transporter permease	*Leuconostoc lactis* 824 *Leuconostoc mesenteroides* 156 *Weissella confusa* 44
cylE	N-acyltransferase	*Leuconostoc lactis* 824
cylI	3-ketoacyl-ACP synthase	Full length match in *Leuconostoc lactis* 824, partial matches in other strains
cylK	Phosphopantetheinyl transferase	*Leuconostoc lactis* 824

Table 9 LAB strains containing partial homologues to internalin genes of *Listeria monocytogenes*

	Coverage of internalin A (%)	Amino acid identity against internalin A (%)
Weissella confusa 44	59	32
Weissella confusa 744	76	34
Weissella cibaria 22	64	34
Weissella cibaria 752	65	34
Weissella cibaria 758	65	34
Leuconostoc holzapfelii 733	53	31
Leuconostoc lactis 824	56	34
Lactococcus lactis 537	72	26

Table 10 Antibiotic resistance genes annotated in RAST.

[a]Commercially available probiotic strains were added for comparison

parC = topoisomerase IV subunit A; parE = topoisomerase IV subunit B; gyrA, gyrB = DNA gyrase subunit A and B; Lde =efflux pump Lde; BL = β-lactamase; *BLc = β-lactamase class C and other penicillin binding proteins; BLA = β-lactamase class A; MFS = multidrug-efflux transporter, major facilitator superfamily; *Reg = probable transcription regulator protein of MDR efflux pump cluster; MacB = macrolide export ATP-binding/permease protein; PmrA = Multidrug resistance efflux pump PmrA

	Resistance to fluoroquinolones					B-lactamase			Multidrug resistance efflux pumps				
	parC	*parE*	*gyrA*	*gyrB*	Lde	BL	*BLc	BLA	*Reg	*MATE family MDR Pump	MFS	MacB	PmrA
Lactobacillus acidophilus NCFM[a]	yes	yes	yes	yes	yes	no	yes	yes	no	no	no	no	no
Lactobacillus johnsonii NCC 533[a]	yes	yes	yes	yes	no	no	no	yes	no	yes	no	no	no
Lactobacillus plantarum WCFS1[a]	yes	yes	yes	yes	no	yes	yes	yes	no	yes	no	no	no
Lactococcus lactis 21	yes	yes	yes	yes	yes	no	no	no	no	yes	yes	yes	yes
Lactococcus lactis 417	yes	yes	yes	yes	yes	no	no	no	no	yes	yes	no	yes
Lactococcus lactis 537	yes	yes	yes	yes	yes	no	no	no	no	yes	yes	yes	yes
Leuconostoc garlicum 733	yes	yes	yes	yes	no	no	yes	no	no	yes	yes	no	no
Leuconostoc lactis 824	yes	yes	yes	yes	no	no	yes	no	no	yes	yes	no	no
Leuconostoc mesenteroides 109	yes	yes	yes	yes	no	no	yes	no	no	no	yes	no	no
Leuconostoc mesenteroides 156	yes	yes	yes	yes	no	no	yes	yes	no	no	yes	no	no
Leuconostoc mesenteroides 684	yes	yes	yes	yes	no	no	yes	yes	no	no	yes	no	no
Leuconostoc mesenteroides 838	yes	yes	yes	yes	no	no	yes	no	no	no	yes	no	no
Weissella cibaria 22	yes	no	yes	yes	no	no	yes	no	yes	yes	yes	no	no
Weissella cibaria 752	yes	no	yes	yes	no	no	yes	no	no	yes	yes	no	no
Weissella cibaria 758	yes	no	yes	yes	no	no	yes	no	yes	yes	yes	no	no
Weissella confusa 44	yes	yes	yes	yes	no	no	yes	no	yes	yes	yes	no	no
Weissella confusa 744	yes	yes	yes	yes	no	no	yes	no	yes	yes	yes	no	no
Weissella soli 498	yes	yes	yes	yes	no	no	no	no	no	yes	yes	no	no

Table 11 Positive control sequences used for testing RGI

Bacterial strain	Antimicrobial drug class	Mechanism of resistance	Antimicrobial resistance determinant	Accession no.	Reference
Pathogens					
Staphylococcus aureus M92	acridine dyes; fluoroquinolones	antibiotic efflux	*arlR* *arlS*	CP015447	McClure and Zhang (2017)
	acridine dyes; penams; tetracyclines; peptide antibiotics; cephalosporins; fluoroquinolones	antibiotic efflux	*mgrA*		
	aminoglycosides	antibiotic inactivation	AAC(6')-IeAPH(3')-IIIa APH(2'')-Ia		
	fosfomycin	antibiotic target alteration	*murA* mutation (G257D)		
	nucleoside antibiotics	antibiotic inactivation	SAT-4		
	penams	antibiotic inactivation	*blaZ*		
	penams; monobactams; carbapenems; cephamycins; cephalosporins	antibiotic target replacement	*mecA* *mecI* *mecR1*		
	streptogramins; macrolides; lincosamides	antibiotic target alteration	*ermA*		
	tetracyclines	antibiotic efflux	*tet*(38) *tet*(K)		
		antibiotic target protection	*tet*(W/N/W)		
	tetracyclines; glycylcyclines	antibiotic efflux	*mepA* *mepR*		
	multi-drug	antibiotic efflux	sav1866		
Staphylococcus aureus COL	acridine dyes; fluoroquinolones	antibiotic efflux	*arlR* *arlS*	NC_002951	Gill et al. (2005)
	acridine dyes; penams; tetracyclines; peptide antibiotics; cephalosporins; fluoroquinolones	antibiotic efflux	*mgrA*		
	penams; monobactams; carbapenems; cephamycins; cephalosporins	antibiotic target replacement	*mecA* *mecR1*		
	tetracyclines	antibiotic efflux	*tet*(38)		

	tetracyclines; glycylcyclines	antibiotic efflux	*mepA* *mepR*		
	multi-drug	antibiotic efflux	sav1866		
Enterococcus faecalis DS16, transposon TN916	tetracyclines	antibiotic target protection	*tet*(M)	U09422	Flannagan et al. (1994)
Enterococcus faecium Aus0004	aminoglycosides	antibiotic efflux	AAC(6')-Ii	NC_017022	Lam et al. (2012)
	diaminopyrimidines	antibiotic target replacement	*dfrG*		
	fluoroquinolones; macrolides	antibiotic efflux	*efmA*		
	glycopeptides	antibiotic target alteration	*vanXB* *vanSB* *vanHB* *vanB* *vanWB* *vanYB* *vanRB*		
	macrolides; streptogramins	antibiotic efflux	*msrC*		
	macrolides; streptogramins; lincosamides	antibiotic target alteration	*ermG*		
	tetracyclines	antibiotic target protection	*tet*(W/N/W)		
Enterococcus faecalis V583	acridine dyes	antibiotic efflux	*emeA*	NC_004668	Paulsen et al. (2003)
	diaminopyrimidines	antibiotic target replacement	*dfrE*		
	glycopeptides	antibiotic target alteration	*vanXB* *vanSB* *vanHB* *vanB* *vanWB* *vanYB* *vanRB*		
	rifamycins; fluoroquinolones; macrolides	antibiotic efflux	*efrA* *efrB*		
	streptogramins; lincosamides; pleuromutilins	antibiotic efflux	*lsaA*		
Klebsiella pneumoniae DHQP1605752_NV	aminocoumarins; aminoglycosides	antibiotic efflux	*baeR*	CP022127	de Man et al. (2018)
	cephalosporins	antibiotic inactivation	CTX-M-15		

	cephalosporins; monobactams; penems; carbapenems; cephamycins; penams	reduced permeability to antibiotic	OmpK37		
	cephalosporins; penams; carbapenems	antibiotic inactivation	SHV-28		
	diaminopyrimidines; tetracyclines; nitrofurans; fluoroquinolones; glycylcyclines	antibiotic efflux	*oqxA*		
	fluoroquinolones	antibiotic target alteration	*parC* mutation - S80I		
		antibiotic efflux	*emrB* *emrR* *patA*		
	fluoroquinolones; tetracyclines	antibiotic efflux	*adeF*		
	fosfomycin	antibiotic target alteration	*uhpT* mutation - E350Q		
		antibiotic inactivation	*fosA6*		
	macrolides; streptogramins monobactams; carbapenems; rifamycins; penams; triclosan; glycylcyclines; tetracyclines; cephalosporins; phenicols; penems; fluoroquinolones; cephamycins	antibiotic efflux reduced permeability to antibiotic; antibiotic efflux	*msrB* *marA*		
	nitroimidazoles	antibiotic efflux	*msbA*		
	penams; fluoroquinolones; macrolides	antibiotic efflux	*crp*		
	penams; monobactams; carbapenems; cephamycins; cephalosporins	antibiotic target alteration	PBP3 mutations - S357N, D350N		
	penams; tetracyclines; cephalosporins; fluoroquinolones; macrolides; cephamycins	antibiotic efflux	*hns*		
	rifamycins; cephalosporins; triclosan; glycylcyclines; tetracyclines; penams; phenicols; fluoroquinolones	antibiotic target alteration; antibiotic efflux	*marR* mutation; *ramR* mutation – A19V		
		antibiotic efflux	*acrA*		
	triclosan	antibiotic target alteration	*gyrA* mutation - S83F		
Klebsiella pneumoniae DHQP1605752_NV, plasmid p1605752AC2	aminoglycosides	antibiotic target alteration	*rmtC*	CP022126	de Man et al. (2018)
		antibiotic	AAC(6')-Ib7		

		inactivation			
	cephalosporins; penams; carbapenems; cephamycins	antibiotic inactivation	NDM-1		
	cephamycins	antibiotic inactivation	CMY-6		
	glycopeptides	antibiotic inactivation	BRP(MBL)		
	sulfonamides; sulfone antibiotics	antibiotic target replacement	*sul1*		
Klebsiella pneumoniae DHQP1605752_NV, plasmid p1605752FIB	macrolides	antibiotic inactivation	*mrx* *mphA*	CP022125	de Man et al. (2018)
Acinetobacter baumannii AYE	acridine dyes; fluoroquinolones; triclosan	antibiotic efflux	*abeM*	CU459141	Vallenet et al. (2008)
	aminocoumarins; macrolides	antibiotic efflux	*abeS*		
	aminoglycosides	antibiotic inactivation	AAC(3)-Ia ANT(2'')-Ia ANT(3'')-IIa APH(3')-Ia APH(3'')-Ib APH(6)-Id *aadA*		
	cephalosporins; monobactams	antibiotic inactivation	VEB-1		
	cephalosporins; penams	antibiotic inactivation	*ampC* OXA-10 OXA-69		
	diaminopyrimidines	antibiotic target replacement	*dfrA10* *dfrA1*		
	fluoroquinolones; tetracyclines	antibiotic efflux	*adeF* *adeG* *adeH* *adeL*		
	phenicols	antibiotic inactivation	*catI*		
		antibiotic efflux	*cmlA5*		
	rifamycins	antibiotic inactivation	*arr-2*		
	rifamycins; lincosamides; fluoroquinolones; tetracyclines; penems; phenicols;	antibiotic efflux	*adeI* *adeJ*		

	carbapenems; macrolides; cephalosporins; diaminopyrimidines		*adeK* *adeN*		
	sulfonamides; sulfone antibiotics	antibiotic target replacement	*sul1*		
	tetracyclines; glycylcyclines	antibiotic efflux	*adeA* *adeB* *adeC* *adeR* *adeS*		
Acinetobacter baumannii ACICU	acridine dyes; fluoroquinolones; triclosan	antibiotic efflux	*abeM*	CP000863	Iacono et al. (2008)
	aminocoumarins; macrolides	antibiotic efflux	*abeS*		
	aminoglycosides	antibiotic inactivation	AAC(6')-Ib7 ANT(3'')-IIa APH(3')-Ia		
	cephalosporins	antibiotic inactivation	ADC-74		
	cephalosporins; penams	antibiotic inactivation	OXA-66 OXA-20		
	fluoroquinolones; tetracyclines	antibiotic efflux	*adeF* *adeG* *adeH* *adeL*		
	rifamycins; lincosamides; fluoroquinolones; tetracyclines; phenicols; penems; carbapenems; macrolides; cephalosporins; diaminopyrimidines	antibiotic efflux	*adeI* *adeJ* *adeK* *adeN*		
	sulfonamides; sulfone antibiotics	antibiotic target replacement	*sul1*		
	tetracyclines; glycylcyclines	antibiotic efflux	*adeA* *adeB* *adeC* *adeR* *adeS*		
Acinetobacter baumannii ACICU, plasmid pACICU1	cephalosporins; penams	antibiotic inactivation	OXA-58	NC_010605	Iacono et al. (2008)
Mycobacterium tuberculosis H37Rv	aminoglycosides	antibiotic inactivation	AAC(2')-Ic	NC_000962	Cole et al. (1998)
	fluoroquinolones	antibiotic target protection	*mfpA*		

	penams; macrolides	antibiotic efflux	*mtrA*		
	rifamycins	antibiotic target protection	*rbpA*		
	rifamycins; isoniazid	antibiotic efflux	*efpA*		
	streptogramins; macrolides; lincosamides	antibiotic target alteration	*erm*(37)		
Pseudomonas aeruginosa ATCC BAA-2109*	acridine dyes; carbapenems; macrolides; cephalosporins; tetracyclines; penams; phenicols; fluoroquinolones; cephamycins; aminoglycosides	antibiotic efflux	*mexY* *mexZ*	MVGW01000000	Jeukens et al. (2017)
	acridine dyes; diaminopyrimidines; tetracyclines; phenicols; carbapenems; macrolides	antibiotic efflux	*mexP* *mexQ* *opmE*		
	acridine dyes; fluoroquinolones; tetracyclines	antibiotic efflux	*mexG* *mexH* *mexI* *opmD*		
	acridine dyes; tetracyclines; phenicols; fluoroquinolones; macrolides	antibiotic efflux	*mexV* *mexW*		
	aminoglycosides	antibiotic efflux	*emrE*		
		antibiotic inactivation	APH(3')-IIb		
	aminoglycosides; peptide antibiotics; macrolides; tetracyclines; penams; sulfonamides; aminocoumarins; monobactams; carbapenems; acridine dyes; diaminopyrimidines; cephalosporins; phenicols; penems; fluoroquinolones; cephamycins	antibiotic efflux	*oprM*		
	bicyclomycin	antibiotic efflux	*bcr-1*		
	cephalosporins; monobactams; carbapenems	antibiotic inactivation	PDC-5		
	fluoroquinolones; tetracyclines	antibiotic efflux	*adeF*		
	fosfomycin	antibiotic inactivation	*fosA*		
	penams; cephalosporins	antibiotic inactivation	OXA-50		
	penams; fluoroquinolones; aminocoumarins; aminoglycosides; tetracyclines; cephalosporins;	antibiotic efflux	*mexC* *mexD*		

phenicols; macrolides; diaminopyrimidines		*oprJ* Type B *nfxB*
peptide antibiotics	antibiotic target alteration	*arnA* *basR* *basS*
phenicols	antibiotic efflux	*mexM* *mexN*
	antibiotic inactivation	*catB7*
phenicols; diaminopyrimidines; fluoroquinolones	antibiotic efflux	*mexE* *mexS* *mexT* *oprN*
rifamycins; acridine dyes; penams; triclosan; glycylcyclines; tetracyclines; cephalosporins; phenicols; fluoroquinolones	antibiotic target alteration; antibiotic efflux	*soxR*
sulfonamides; aminocoumarins; peptide antibiotics; monobactams; carbapenems; macrolides; diaminopyrimidines; penams; tetracyclines; cephalosporins; phenicols; penems; fluoroquinolones; cephamycins	antibiotic efflux	*mexA* *mexB* *nalC* *nalD* *armR*
sulfonamides; aminocoumarins; peptide antibiotics; monobactams; carbapenems; macrolides; penams; diaminopyrimidines; tetracyclines; cephalosporins; phenicols; penems; fluoroquinolones; cephamycins; aminoglycosides	antibiotic efflux	*cpxR*
sulfonamides; aminocoumarins; peptide antibiotics; monobactams; carbapenems; macrolides; penams; diaminopyrimidines; tetracyclines; cephalosporins; phenicols; penems; fluoroquinolones; cephamycins	antibiotic target alteration; antibiotic efflux	*mexR*
tetracyclines; aminocoumarins; monobactams; macrolides	antibiotic efflux	*muxA* *muxB* *muxC* *opmB*
tetracyclines; triclosan; macrolides	antibiotic efflux	*mexJ* *mexK*

			mexL		
	triclosan	antibiotic efflux	*triA* *triB* *triC* *opmH*		
	multi-drug	antibiotic efflux	*pmpM*		
Klebsiella aerogenes C10*	aminocoumarins; aminoglycosides	antibiotic efflux	*baeR*	LUTZ01000000	Grazziotin et al. (2016)
	aminocoumarins; fluoroquinolones	antibiotic target alteration	*gyrB* mutation - E466D		
	aminoglycosides	antibiotic inactivation	AAC(6')-Ib10 *aadA* AAC(3)-IIc		
	cephalosporins	antibiotic inactivation	CTX-M-2		
	cephalosporins; penams	antibiotic inactivation	OXA-9		
	cephalosporins; penams; carbapenems	antibiotic inactivation	OXA-2		
	cephalosporins; penams; monobactams; penems	antibiotic inactivation	TEM-1 TEM-160		
	diaminopyrimidines; tetracyclines; nitrofurans; fluoroquinolones; glycylcyclines	antibiotic efflux	*oqxA*		
	fluoroquinolones	antibiotic target protection	*qnrS1*		
		antibiotic efflux	*patA* *emrB* *emrR*		
	fluoroquinolones; tetracyclines	antibiotic efflux	*adeF*		
	fosfomycin	antibiotic inactivation	*fosA5*		
		antibiotic target alteration	*uhpT* mutation - E350Q		
	macrolides; streptogramins monobactams; carbapenems; rifamycins; penams; triclosan; glycylcyclines; tetracyclines; cephalosporins; phenicols; penems; fluoroquinolones; cephamycins	antibiotic efflux antibiotic target alteration; reduced permeability to	*msrB* *soxS* mutation		

		antibiotic; antibiotic efflux			
		reduced permeability to antibiotic; antibiotic efflux	*marA*		
	nitroimidazoles	antibiotic efflux	*msbA*		
	nybomycin; fluoroquinolones	antibiotic target alteration	*gyrA* mutation - S83I		
	penams; fluoroquinolones; macrolides	antibiotic efflux	*crp*		
	penams; monobactams; carbapenems; cephamycins; cephalosporins	antibiotic target alteration	PBP3 mutations - S357N, D350N		
	penams; tetracyclines; cephalosporins; fluoroquinolones; macrolides; cephamycins	antibiotic efflux	*hns*		
	phenicols	antibiotic inactivation	*catII*		
	rifamycins; cephalosporins; triclosan; glycylcyclines; tetracyclines; penams; phenicols; fluoroquinolones	antibiotic target alteration; antibiotic efflux	*marR* mutation - S3N		
	streptogramins; pleuromutilins	antibiotic efflux	*vgaC*		
	sulfonamides; sulfone antibiotics	antibiotic target replacement	*sul1*		
Escherichia coli NA114*	acridine dyes; lincosamides; nucleoside antibiotics; phenicols; fluoroquinolones	antibiotic efflux	*mdtM*	MIPU01000000	Avasthi et al. (2011)
	acridine dyes; nucleoside antibiotics	antibiotic efflux	*mdtN* *mdtO* *mdtP*		
	aminocoumarins	antibiotic efflux	*mdtA* *mdtB* *mdtC*		
	aminocoumarins; aminoglycosides	antibiotic efflux	*cpxA* *baeR* *baeS*		
	aminocoumarins; macrolides; rifamycins; penams; triclosan; glycylcyclines; tetracyclines; cephalosporins; phenicols; fluoroquinolones; cephamycins	antibiotic efflux	*tolC*		
	aminoglycosides	antibiotic inactivation	*aadA5*		

	antibiotic efflux	*kdpE* *acrD*
cephalosporins	antibiotic inactivation	CTX-M-15
cephalosporins; penams	antibiotic inactivation	OXA-1 *ampC*
cephalosporins; penams; fluoroquinolones; cephamycins	antibiotic efflux	*acrE* *acrF*
diaminopyrimidines	antibiotic target replacement	*dfrA17*
fluoroquinolones	antibiotic efflux	*emrA* *emrB* *emrR* *patA* *mdtH*
	antibiotic target alteration	*parC* mutation - S80I
fluoroquinolones; aminoglycosides	antibiotic inactivation	AAC(6')-Ib-cr
fosfomycin	antibiotic target alteration	*ptsI* mutation - V25I *uhpT* mutation - E350Q *glpT* mutation - E448K
	antibiotic efflux	*mdtG*
macrolides	antibiotic efflux	*emrE*
	antibiotic inactivation	*mrx* *mphA*
macrolides; streptogramins monobactams; carbapenems; rifamycins; penams; triclosan; glycylcyclines; tetracyclines; cephalosporins; phenicols; penems; fluoroquinolones; cephamycins	antibiotic efflux reduced permeability to antibiotic; antibiotic efflux	*msrB* *marA*
monobactams; carbapenems; rifamycins; penams; triclosan; glycylcyclines; tetracyclines; cephalosporins; phenicols; penems; fluoroquinolones; cephamycins	antibiotic target alteration; reduced permeability to antibiotic; antibiotic efflux	*soxS* mutation

nitroimidazoles	antibiotic efflux	*msbA*
nybomycin; fluoroquinolones	antibiotic target alteration	*gyrA* mutations - S83L, D87N,
penams; fluoroquinolones; macrolides	antibiotic efflux	*crp* *gadX* *gadW* *mdtE* *mdtF*
penams; monobactams; carbapenems; cephamycins; cephalosporins	antibiotic target alteration	PBP3 mutations - S357N, D350N
penams; tetracyclines; cephalosporins; fluoroquinolones; macrolides; cephamycins	antibiotic efflux	*hns*
peptide antibiotics	antibiotic efflux	*yojI*
	antibiotic target alteration	*pmrC* *pmrE* *arnA* *bacA*
phenicols	antibiotic inactivation	*catB3*
rhodamine; tetracyclines; benzalkonium chloride	antibiotic efflux	*mdfA*
rifamycins; cephalosporins; triclosan; glycylcyclines; tetracyclines; penams; phenicols; fluoroquinolones; cephamycins	antibiotic efflux	*acrS*
rifamycins; cephalosporins; triclosan; glycylcyclines; tetracyclines; penams; phenicols; fluoroquinolones	antibiotic target alteration; antibiotic efflux	*marR* mutations - G103S, Y137H *acrR* mutation *soxR* mutation
	antibiotic efflux	*acrA* *acrB*
streptogramins; pleuromutilins sulfonamides; sulfone antibiotics	antibiotic efflux antibiotic target replacement	*vgaC* *sul1*
tetracyclines	antibiotic efflux	*emrK* *emrY*
tetracyclines; penams; fluoroquinolones; macrolides	antibiotic efflux	*evgA* *evgS*
multi-drug	antibiotic efflux	*emrD*

Non-pathogenic LAB					
Lactobacillus plantarum MF345, plasmid pLFE1	erythromycin	antibiotic target alteration	*ermB*	FJ374272	Feld et al. (2009)
Lactobacillus sakei Rits9, plasmid pLS55†	tetracyclines	antibiotic efflux	*tetL*	EF605268	Ammor et al. (2008)
Lactobacillus sakei Rits9, transposon-associated sequence	tetracyclines	antibiotic target protection	*tetM*	EF605269	Ammor et al. (2008)
Lactobacillus reuteri SD2112, plasmid pLR581	tetracyclines	antibiotic target protection	*tetW*	NC_015700	Biogaia website‡; Rosander et al. (2008)
Lactobacillus reuteri SD2112, plasmid pLR585	lincomycin	antibiotic inactivation	*lnuA*	NC_015698	Biogaia website‡; Rosander et al. (2008)
Leuconostoc mesenteroides subsp. *dextranicum* LbE15*	erythromycin	antibiotic target alteration	*ermB*	LAYN01000000	(Campedelli et al., 2015; Flórez et al., 2016)
Leuconostoc mesenteroides subsp. *mesenteroides* LbE16*	aminoglycosides	antibiotic inactivation	*aadE(ant6)* *sat4* *aphA-3* *mmr*	LAYU01000000	(Campedelli et al., 2015; Flórez et al., 2016)
	streptogramins (virginiamycin)	antibiotic inactivation	*vatE* (*satG*)		
	tetracyclines	antibiotic target protection	*tetS*		
Lactobacillus reuteri 100-63, plasmid pGT633	erythromycin	antibiotic target alteration	*ermGT*	M64090	Tannock et al. (1994)
Lactobacillus fermentum ROT1, plasmid pLME300	macrolide-lincosamide-streptogramin B	antibiotic target alteration	*ermLF*	AJ488494	Gfeller et al. (2003)
	streptogramin A	antibiotic inactivation	*vatE*		
Lactobacillus reuteri PA-16	erythromycin	antibiotic target alteration	*ermC*	FJ489650	Egervarn et al. (2009)
Lactobacillus reuteri G4, plasmid pTC82	chloramphenicol	antibiotic inactivation	*cat*	NG_047562	Lin et al. (1996)
Lactobacillus johnsonii G41	tetracyclines	antibiotic target protection	*tet*(O/W/32/O/W/O)	DQ525023	van Hoek et al. (2008)

2. Evaluation of commercial probiotic delivery using vegetables

2.1. Addition of commercial probiotics into baby spinach

Commercial probiotic strains in the form of freeze-dried powder provided by two leading probiotic supply companies were added into ready-to-eat baby spinach leaves which were purchased from Coles. Probiotic powder of each strain (15-40 g) was rehydrated in 3 L of tap water for approximately 1 h at room temperature. This 3 L probiotic suspension was used to soak 4 batches of 250 g of baby spinach for 5 min each batch, resulting in an initial concentration of 10^7-10^8 CFU/g. Spinach leaves were then dried in a salad spinner for 2 min, split into 120 g aliquots in BOPP film bags and sealed under air conditions. The bags of probiotic spinach leaves were stored at 4 °C. A control sample without probiotic addition was prepared in the same way. Enumeration of probiotic counts was conducted on days 0, 3 and 7 during storage period by spread plating ten-fold dilutions of spinach samples in 0.1% peptone water on MRS agar.

2.2. Preparation of probiotic stocks for testing with salad dressing and simulated gastric and intestinal juice

Single colonies of the six probiotic strains mentioned in Section 2.1.2 in Methodology obtained on MRS agar were inoculated into MRS broth and incubated overnight to reach stationary phase. The cells were pelleted by centrifugation at 5000 × g for 10 min at 20 °C and washed twice in 0.85% NaCl. The washed cells were resuspended in 10% skimmed milk to 10^9-10^{10} CFU/mL. These concentrated stocks were stored at -80 °C and used to inoculate baby spinach and skimmed milk as described below.

2.2.1. Survival of probiotics in baby spinach in presence of salad dressing

Seventy-five grams of baby spinach were placed in sealed bags and sterilised under UV light for 5 min on each side. A 1.5 mL aliquot of frozen probiotic stock of each strain prepared in Section 2.2 was dissolved in 150 mL of sterile water and used to soak the baby spinach for 5 min. The baby spinach was then dried in a salad spinner for 2 min and divided into 15 g aliquots each in 5 stomacher bags. The spinach was incubated at 4 °C for 1 h. Subsequently, 5 mL of each type of salad dressing was added to 3 bags of spinach respectively. The remaining 2 bags of salad acted as controls: 5 mL of water was added to one and the other had no further addition. The spinach was then incubated at room temperature for 15 min. After that, the spinach in each bag was homogenised in 85 mL (spinach only control) or 80 mL (others) of 0.1% peptone water. Further ten-fold serial dilutions were prepared and used for spread plating to obtain bacterial counts.

2.2.2. Survival of probiotics in presence of simulated gastric and intestinal juice

Ten grams of baby spinach were weighed into sterile bags and sterilised under UV light for 5 min on each side. Afterwards, the spinach was inoculated with the tested probiotic strain stock prepared in Section 2.2 diluted in sterile deionised water to result in a final concentration of 10^7 CFU/g. A control was prepared in which the spinach was replaced 10 mL of skimmed milk containing 10^7 CFU/mL of probiotic. The samples were left for 5 min to ensure homogenous distribution of bacteria and then dried with a salad spinner for 2 min. Subsequently, the spinach and skimmed milk was transferred into a stomacher bag respectively and stored at 8 °C for 24 h. The following day, 90 mL of simulated gastric juice or simulated intestinal juice was added to each sample and incubated at 37 °C for 2 hours. These juices were prepared based on the methods of Liong and Shah (2005). Gastric juice was simulated with 0.1% peptone water adjusted to a pH of 2.0 with 1.0 M HCl. Simulated intestinal juice was prepared by

supplementing 0.1% peptone water with 0.3% (w/v) oxgall bile salt and adjusting the pH to 6.0 with 0.1 M HCl. Samples were taken every 30 min (gastric juice) or every hour (intestinal juice) for enumeration.

2.3. Sensory evaluation of probiotic baby spinach

The study was advertised and invited expressions of interest for the sensory trials. People who expressed their interest to participate in the study were explained about the project and informed about the presence of potential allergens (e.g. lactose, milk proteins). Forty people without food intolerances and compromised immune system participated in the sensory trial after signing a consent form. Probiotic spinach samples were evaluated for sensory quality using a triangle taste test. The un-trained panellists were given three different samples of spinach leaves prepared without probiotic or with probiotic strain A or strain B and each labelled with a three-digit code. Two samples were the same and one was different. The six possible order combinations were randomised across the panellists. Each sample was assessed in the order provided, from left to right, and the assessors were asked to select the sample that was different from the other two. The sensory evaluation form is presented as below.

SENSORY EVALUATION FORM

Product: Baby spinach leaves_Set A
Assessor No.: Date: 29 Jan, 2018

(Note: Samples may contain milk. Please do not participate in this study if you are allergic to milk.)
Instructions:

Please rinse your mouth with water provided before starting the test and between samples.

You are provided with three samples of baby spinach leaves, each labeled with a three-digit code. Two of these three samples are identical while the third is odd or different.

1. Taste the samples in the order provided, **from left to right**, and identify the odd sample by placing a **TICK** (✓) for the code corresponding to the **ODD** sample.

You may retaste the samples.

Sample code	219	624	763
Check the odd sample (please tick)			

2. Indicate the degree of difference between the duplicate samples and the odd sample.

Slight _____
Moderate _____
Large _____
Extreme _____

3. Acceptability

Odd sample more acceptable _____
Duplicate samples more acceptable _____

4. Please try to describe the reason why the odd sample is different.

_____

_____

Thank you for your participation.

Appendix B: Certificate of analysis

CERTIFICATE OF ANALYSIS

ANALYSIS PERFORMED FOR
Van Ho
University of QLD - School of Ag & Food
The University of QLD
St Lucia QLD 4072

CERTIFICATE NO.: B633000
ISSUE DATE: 23/01/18
REVISION NO: 01
This certificate supercedes any previous revisions

JOB INFORMATION
Description: Fruit & Veg - Spinach
Date Received: 18/01/2018 12:30
Testing Commenced: 18/01/2018
Order No:
Sample Info: Fruit & Veg - Spinach

CONDITIONS OF SAMPLE ON RECEIPT
Receipt Temperature: 4.6 ℃ (Surface Temperature taken by infra-red)
Storage Temperature: 4 ℃

RESULTS OF ANALYSIS
Sample(s) were analysed as received, and the results pertain only to the submitted sample(s).

CODE	Sample DESCRIPTION	Escherichia coli CFU/g	CP Staphylococci CFU/g	Salmonella spp. /25g	Listeria monocytogenes /25g
B633000-1	Test:19/01/2018, Spinach BB-12 120g	<10	<100	ND	ND
B633000-2	Test:19/01/2018, Spinach Control 120g	<10	<100	ND	ND
		M8.8 AOAC 991.14	M18.1 AS 5013.12.1-2004	M16.7 Salmonella by SOLUS	M13.7 Listeria by SOLUS

DEFINITIONS: > = Greater than < = Less than ~ = Estimated ND = Not detected MPN = Most probable number CFU = Colony forming units
- = Not tested TBA = To be advised * = Test not covered by scope of NATA accreditation RP = Result Pending, confirmatory testing in progress

Rossini (Rose) Elias, Senior Laboratory Technician
Microbiology (Brisbane)

Page 1 of 1

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- Melbourne: Unit 15, 640-680 Geelong Rd, Brooklyn VIC 3025
- Melbourne: 2/1G Marine Parade, Abbotsford VIC 3067
- Sydney: 2 Sirius Rd, Lane Cove West NSW 2066
- Rockhampton: Unit 3, 191 Berserker St, Berserker QLD 4701
- Wagga Wagga: Unit 5, 10-12 Kooringal Rd, Wagga Wagga NSW 2650
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Symbio LABORATORIES

CERTIFICATE OF ANALYSIS

CERTIFICATE NO.: B668587 **REVISION NO.:** 00
ISSUE DATE: 18/05/2018 This certificate supersedes any previous revisions

CLIENT DETAILS: Van Ho
University of QLD - School of Ag & Food
The University of QLD
St Lucia QLD 4072
JOB DESCRIPTION: Fruit & Veg - Lettuce
CLIENT REF:
DATE RECEIVED: 09/05/2018
TEST DATE: Sample tested between date received and reported.

CONDITIONS OF SAMPLE: Receipt Temperature: Chilled (0 ~ 5 °C)
Storage Temperature: Refrigerated

RESULTS OF ANALYSIS:

Sample Description Test	Method Code	Unit	B668587-1 109	B668587-2 744	B668587-3 752
CR107_Biogenic Amine_MBM					
beta-Phenylethylamine (free amine)	CR107	mg/kg	<2	<2	<2
Putrescine (free amine)	CR107	mg/kg	19	20	18
Cadaverine (free amine)	CR107	mg/kg	<2	5.3	2.3
Histamine (free amine)	CR107	mg/kg	<2	<2	<2

DEFINITIONS: < : Less than, > : Greater than, - : Not Tested, DWB : Dry Weight Basis.
* : The test is not covered by the scope of accreditation.

: The result is derived from a calculation incorporating the residue definition of analytes reported as defined in Schedule 20 and Schedule 21 of Standard 1.4.2 – Agvet Chemicals. Only results above the LOR are included in the calculation.

Results were reported on an "as received" basis unless otherwise indicated.
Sampling was conducted by the customer and results reported pertain only to the samples submitted.
Responsibility for representative sampling rests with the customer.

Eddie Wang, Senior Laboratory Technician
Chemistry (Brisbane)

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Appendix C: Dossier prepared for FSANZ

Lactic acid bacteria are ideal candidates for application as biocontrol agents, because they are non-pathogenic, have a long history of safe use as food preservatives and are thus classed as Generally Regarded As Safe (GRAS). LAB are found in a wide range of fresh unprocessed foods (e.g. fresh fruits and vegetables), many fermented foods (e.g. yoghurt, cheese, sauerkraut, kimchi) and are marketed as probiotics. Raw vegetables and fruits contain generally low levels of LAB (10^2 to 10^4 CFU/g) out of a total bacterial population of around 10^5 to 10^7 CFU/g (Di Cagno et al., 2013). Many fermented foods (e.g. dairy, vegetable, cereal and meat based) however are enriched in LAB which can be present at levels of 10^8 CFU/g and higher. For example, the Korean fermented cabbage food kimchi contains $5x10^8$ LAB per ml (Cho et al., 2006). Fermentation of foods or the addition of LAB to foods can be carried out for variety of reasons such as to increase the shelf-life (preservation), improve the organoleptic properties (flavour/aroma) or for health promotion (probiotic effects).

A recent review of microorganisms with a history of use in foods was carried out by a taskforce representing the International Dairy Federation (IDF) and the European Food and Feed Cultures Association (EFFCA) (Bourdichon et al., 2012). A list of 264 "microbial species with technical benefit for food fermentation" was published in 2012 (IDF, 2012). On the list are the following bacteria: *Lactococcus*, *Lactobacillus*, *Leuconostoc* and *Weissella*, which all have a long safe history of use in food fermentation and/or as probiotics. Indeed *Lactobacillus*, *Leuconostoc* and *Weissella* are dominant LAB found in kimchi (Table 12) are thus consumed live and in high doses in fresh kimchi by millions each day. *Lactococcus* is the primary starter culture bacterium used in cheese making and also would be consumed in high doses by millions each day also. Therefore, these bacteria pose extremely low risk to human health following consumption even at high levels.

As well as their presence in foods, LAB are regularly found in mucosal surfaces of humans and animals including the mouth, gastrointestinal tract and female urogenital tract. Despite their consumption by millions of people daily and their presence on mucosal surfaces, *Lactococcus*, *Lactobacillus*, *Leuconostoc* and *Weissella* are very rarely associated with clinical disease in humans (Bourdichon et al., 2012). Links between these infections and consumption of the food have not been reported and therefore the causative agent is likely to be present on the person as a commensal organism (Bourdichon et al., 2012). In a review of rare *Lactobacillus* infections, underlying disease or immunosuppression in the patient almost always occurs (Aguirre and Collins, 1993). An estimate of the infections caused by *Lactobacillus* in France is extremely low, at about one case per 10 million people (Bernardeau et al., 2006). Very rare cases of bacteremia caused by *Weissella* have been reported following surgical procedures, likely due to entry of mucosal surface commensal *Weissella* into the blood (Kamboj et al., 2015). *Lactococcus lactis* and *Leuconostoc* have been the cause of just 2-3 reported cases of endocarditis (Adams, 1999). There is no evidence to suggest that foodborne LAB initiate infections, but rather it is commensal LAB that have caused extremely rare complications and only in immunocompromised people.

Table 12 Relative proportions (%) of LAB species in laboratory and commercial kimchi samples (Cho et al., 2006)

Species	Microbial isolates (%)									
	A (n = 117)	B (30)	C (202)	D (30)	E (92)	F (36)	G (70)	H (106)	I (30)	J (120)
Lc. carnosum	–	–	–	–	2	–	–	–	–	–
Lc. citreum	3*	63	55	–	16	11	–	2	–	68
Lc. gasicomitatum	67	–	4	–	8	–	–	–	–	–
Lc. gelidum	14	13	–	–	7	–	–	12	–	–
Lc. inhae	5	–	–	–	–	–	–	–	–	–
Lc. kimchii	–	3	–	7	–	–	–	–	–	–
Lc. lactis	5	3	6	–	–	–	–	–	–	–
Lc. mesenteroides	–	7	18	10	8	14	–	–	20	–
W. cibaria	–	10	17	–	2	3	–	–	–	3
W. koreensis	4	–	–	60	7	3	–	–	–	–
W. paramesenteroides	–	–	–	–	–	–	–	–	2	–
W. soli	1	–	1	–	–	–	–	–	–	–
Lb. brevis	–	–	–	–	–	–	–	–	3	10
Lb. curvatus	–	–	–	–	16	47	–	29	43	11
Lb. mali	–	–	–	–	4	–	–	–	–	–
Lb. paraplantarum	–	–	–	–	1	–	–	3	–	–
Lb. pentosus	–	–	–	–	–		–	5	–	–
Lb. plantarum		–	–	–	3	8	3	2	–	–
Lb. sakei	2	–	1	23	26	14	92	50	33	8

Lc., Leuconostoc; W., Weissella; Lb., Lactobacillus.
*% composition of bacterial isolates from kimchi samples.

Food Standards Australia and New Zealand (FSANZ) is currently reviewing a proposed revision of nutritive substances and novel foods. FSANZ plans to develop a list of microorganisms with a known history of safe use. This approach is in agreement with the introduction of a list of microorganisms with a Qualified Presumption of Safety (QPS) by the European Food Safety Authority (EFSA) in 2007. FSANZ is considering to use the QPS list as the basis for a specific list of microorganisms that are eligible as foods. Under this proposal, microorganisms listed in the QPS must be unambiguously identifiable and cultured to maintain genetic stability. It is required to demonstrate that microorganisms do not produce food poisoning toxins and do not contain antibiotic resistance genes (FSANZ, 2017). Several stakeholders submitted feedback on the proposal and supported the use of microorganisms with a history of safe use as quoted below.

"Nestlé supports the grandfathering of live food culture microorganisms. Nestlé supports the concept of recognition of microorganisms added for a purpose other than food culturing provided they have a history of safe use".

"Dairy Australia would support grandfathering of microorganisms that have been intentionally added to foods or ingredients that have been manufactured or sold on Australia or New Zealand at the time of gazettal. Whilst the microorganism may be grandfathered, manufacturers using them will retain the responsibility for ensuring that they are suitable and have a history of safe use."

"Danone supports maintaining the status quo of permissions for all microorganisms used across foods when there has been a safe history of use and no evidence of market failure."

The Australian Beverages Council "also support the view that fermentative and flavour producing food culture microorganisms are inherently safe and have a history of safe use. Therefore, grandfathering of these products is appropriate".

"Dietitians Association of Australia DAA agrees that the use of micro-organisms in food is likely to increase in the future and clarification around their use would be beneficial for manufacturers, regulatory agencies and the public. DAA is supportive of the proposed Grandfathering approach."

"Fonterra could tentatively support the grandfathering approach suggested for all foods and food ingredients produced with and containing live food cultures manufactured in, or sold in ANZ. Fonterra supports the status quo of provisions permitting the use of lactic acid bacteria, for food culture or other purpose (e.g. probiotic), in food categories. We agree microorganisms with a safe history of use do not raise safety concerns."

In our study, the most promising *Probisafe* strains inhibiting *S.* Typhimurium and *L. monocytogenes* have been identified to species level using molecular biology techniques and they belong to the following species: *Weissella cibaria*, *Weissella confusa*, *Weissella soli*, *Leuconostoc mesenteroides*, *Leuconostoc pseudomesenteroides*, *Leuconostoc holzapfelii*, *Lactococcus raffinolactis* and *Lactococcus lactis* (as presented in the report MS103). These species are in the QPS list of EFSA (Ricci et al., 2018) and the IDF-EFFCA inventory of microbial species with technological beneficial role in fermented food products (IDF, 2012). *Weissella* spp. was introduced in 1993 for some species previously classified as *Leuconostoc mesenteroides* and have been used in fermentations of fish, meat, cocoa, vegetables and sourdough (Bjorkroth et al., 2002; Collins et al., 1993; Katina et al., 2009). *Lactococcus lactis* strains are the primary LAB components of starter cultures used for the production of fermented dairy products (Ayad et al., 1999; Morales et al., 2003) and *Lactococcus raffinolactis* is occasionally involved in cheese ripening (Ouadghiri et al., 2005). For the genus *Leuconostoc*, *L. mesenteroides* and *L. pseudomesenteroides* have been used in dairy (Lazos et al., 1993; Sengun et al., 2009) while *L. holzapfelii* is a useful species for coffee fermentation (De Bruyne et al., 2007).

In summary, naturally occurring harmless bacteria isolated from fruits and vegetables in our project belong to microbial species with a history of safe use and have been used in many food products and scientific publications. They are also consistent under the lists of microorganisms with a QPS introduced by the European Food Safety Authority. Therefore, they meet eligible food criteria under the proposal of nutritive substances and novel foods raised by FSANZ.