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Running head: Antifungal activity of lactic acid bacteria on cheese spoilage moulds.

Key words: Antifungal, lactic acid bacteria, *Penicillium*, cheese spoilage, biopreservative

Abstract

Moulds are the most common cheese spoilage organisms which can lead to economic loss as well as raising public health concerns due to the production of mycotoxins. In this study, 897 lactic acid bacteria (LAB) isolated from different herbs, fruits and vegetables were screened for their antifungal activity in an agar plate overlay assay. Thirty-six isolates had weak activity, 11 had moderate activity and 12 were confirmed as having strong activity. The strong antifungal isolates were obtained from a range of different sources but were all identified by 16S rDNA sequencing as being *Lactobacillus plantarum*. The antifungal spectra for these 12 isolates were determined against eight other moulds commonly associated with cheese spoilage and all isolates were found to possess inhibition against *Penicillium solitum*, *Aspergillus versicolour* and *Cladosporium herbarum*, but not against *Penicillium roqueforti*, *Penicillium glabrum*, *Mucor circinelloides*, *Geotricum candidum* or *Byssochlamys nivea*. The absence of sodium acetate from MRS agar resulted in no inhibition of *P. commune*, suggesting the synergistic effect of acetic acid with the antifungal LAB, similarly to that previously reported. To determine their potential as biopreservatives in cheese, LAB isolates were inoculated into cottage cheese prior to the addition of *P. commune*. All *Lb. plantarum* isolates were found to prevent the visible growth of *P. commune* on cottage cheese by between 14 to >25 days longer than cottage cheese that contained either no added LAB or LAB that did not have antifungal activity (*Lactococcus lactis*, *Weisella soli*, *Leuconostoc inhae* and *Leuconostoc mesenteroides* isolates). The results of this study shows that LAB isolated from various herbs, fruits and vegetables possess antifungal activity and have potential for use as biopreservatives in cheese.

1. Introduction

Fungal food spoilage is one of the main causes of food and economical loss all over the world. Despite the dry climate and advanced technology, food loss due to fungal spoilage in Australia is estimated to be more than $10,000,000 per annum, and it would be expected that this level would be significantly higher in more humid countries with less developed technology (Pitt & Hocking, 2009). Although fungi such as yeast and moulds have been used in the processing of many cheese and fermented products (Marth & Yousef, 1991) they are also responsible for the spoilage of many processed dairy foods (Fente-Sampayo et al., 1995).

According to Pitt and Hocking (2009), cheese is very susceptible to the growth of mould, which makes it unsuitable for sale and consumption. Some of these spoilage moulds in cheese may also produce mycotoxins, such as Sterigmatocystin produced by *Aspergillus versicolor,* which could have serious consequences on consumers' health (Northolt, van Egmond, Soentoro & Deijll, 1980). The growth of mould in cheese is largely contributed by mould's ability to grow at refrigeration temperature, low oxygen concentrations, low pH and low water activity. These moulds are also often resistant to the preservative action of free fatty acids and have lipolytic activity that allows them to cause spoilage in cheese.

Various techniques have been applied to inhibit mould growth in retail cheese. Since most mould spores are killed by pasteurization of milk (Doyle & Marth, 1975), it is important to prevent the recontamination and growth of mould. Modified atmosphere packaging (MAP) has been used successfully to retard or prevent mould growth, and other treatments such as the use of chemical preservatives: sorbates, propionate and natamycin have also been applied as mould inhibitors (Ledenbach & Marshall, 2009). However, these techniques do not show complete effectiveness as the ideal gas composition in MAP may vary from different varieties of cheese and the use of sorbates may lead to a “kerosene” flavour defect after being decaboxylated by some fungi (specifically *Penicillium* species) (Liewen, 1992; Marth & Yousef, 1991). Besides the negative effects on taste and flavour, consumers are also becoming more concerned over the use of preservatives and chemicals in food, driving demand towards natural and organic products. Therefore, there is a significant interest to develop natural preservatives to enhance or replace chemical treatments.

Biopreservation or biocontrol refers to the use of natural or controlled microflora, or its antibacterial products to extend the shelf life and enhance the safety of foods (Stiles, 1996). Since lactic acid bacteria (LAB) occur naturally in many food systems and have a long history of safe use in fermented foods, thus classed as Generally Regarded As Safe (GRAS), they have a great potential for extended use in biopreservation. Multiple publications have identified the antibacterial (O’Sullivan, Ross & Hill, 2002) and antifungal (Crowley, Mahony, & van Sinderen, 2013b; Dalié, Deschamps & Richard-Forgot, 2010; Magnusson, Ström, Roos, Sjögren, & Schnürer, 2003; Schnürer & Magnusson, 2005) activities of LAB. The antimicrobial activity of LAB has been credited to the production of several antimicrobial substances such as lactic acid, which lowers the pH in food and helps inhibit the growth of other microorganisms (Brul & Coote, 1999). Other compounds such as hydrogen peroxide, acetic acid, reuterin, diacetyl and bacteriocins also contribute to its preserving capabilities (Caplice & Fitzgerald, 1999; Lindgren & Dobrogosz, 1990). LAB are commonly found on fresh fruits and vegetables and these sources provide a potential source of new antimicrobial strains. LAB from these sources have been investigated for anti-*Listeria* (Allende et al., 2007; Trias, Babosa, Montesinos & Bañeras, 2008) and anti-mould activity (Sathe, Nawani, Dhakephalkar & Kapadnis, 2007; Trias, Bañeras, Montesinos & Badosa, 2008) however only limited work has been carried out specifically investigating the usefulness of LAB in controlling fungal growth in cheese (Garcha, & Natt, 2012; Muhialdin, Hassan, & Sadon, 2011; Schwenninger & Meile, 2004; Zhao, 2011)

This study aims to identify and investigate antifungal activity of LAB isolated from herbs, fruits and vegetables against moulds commonly associated with cheese spoilage, with a focus on *Penicillium commune* which is a major cheese spoilage mould (Hocking, 1994). Those LAB with anti-mould activity were then tested for their abilities to prevent mould growth when applied to cottage cheese, with the future perspective of using them in cheese preservation.

2. Materials and Methods

2.1. Bacteria and mould strains and media.

Eight hundred and ninety seven LAB isolated from fresh herbs, fruits and vegetables (see Supplementary data) purchased from local supermarkets, grocers, markets and farms were isolated using de Mann Rogosa Sharpe (MRS; Oxoid Ltd) agar and incubation at 30°C for 3 days under anaerobic conditions (AnaeroGen system; Oxoid Ltd.). They were then stored at -80°C in MRS broth supplemented with 40% glycerol. For the overlay assay (see below), two different media were evaluated: MRS and modified MRS without sodium acetate (mMRS). The bacteria were inoculated on agar plates and incubated for 48 hours at 30°C under anaerobic conditions.

Nine mould species, *P. commune* (FRR no. 4117), *Penicillium roqueforti* (FRR no. 0058), *Penicillium glabrum* (FRR no. 4190), *Penicillium solitum* (FRR no. 4195), *Geotricum candidum* (FRR no. 4204), *A. versicolor* (FRR no. 0038), *Mucor circinelloides* (FRR no. 4846), *Byssochlamys nivea* (FRR no. 4376), *Cladosporium herbarum* (FRR no. 4199) were obtained from CSIRO FRR culture collection (CSIRO, North Ryde, Australia). Fungal inocula were prepared by resuspending the freeze dried lyophilized mould cultures in distilled water and growing them on malt extract agar (MEA, Difco™) slants at 25°C for 7 days (or until sporulation). Spores were collected by vigorously shaking slants after adding sterile peptone water (0.2% w/v). The concentration of the *P. commune* inocula were adjusted with sterile peptone water (0.2% w/v) to an absorbance of 0.5 at 600nm using a spectrophotometer which resulted in a spore number of ~1 x 10^6 spores/ml.

2.2. Screening of LAB for antifungal activity.

Antifungal activity assay was carried out using the overlay method described by Rouse et al. (2008) with slight modification. All 897 LAB isolates were tested for their antifungal activity against *P. commune* as an initial screening step. *P. commune* was selected as the indicator mould due to its common occurrence as spoilage mould on cheese (Hocking, 1994). Initial screening was carried out by spotting 2µl of 18 LAB isolates from the frozen stock on one MRS agar plate. The plates were incubated anaerobically at 30°C for 48 hours. The plates were then overlaid with 6ml of malt extract soft agar (1.5% malt extract, 0.7% agar; Difco™) containing 0.1ml (1 x 10^6 spores/ml) of *P. commune* and incubated aerobically at room temperature (~25°C) for 3-4 days or until a uniform layer of mould growth was observed. Zones of inhibition around the LAB spots were recorded according to the following scale: (-) no inhibition, colonies are entirely covered by mould, (+) weak inhibition seen on the LAB colony but no distinct clearing zone near the LAB colony, (++) moderate inhibition with small clearing zone near the LAB colony and (+++) strong inhibition with a large zone of clearing around the LAB colony.

LAB isolates that displayed strong inhibition in the preliminary screening were selected for confirmation using the same overlay method. Two LAB isolates with antifungal activity and one LAB isolate that displayed no antifungal activity (negative control) were spotted as above but on a single MRS agar plates and zones of inhibition were recorded according to that above.

2.3. Screening of LAB antifungal activity on modified MRS.

LAB isolates selected for confirmation screening were also tested using the same overlay method on modified MRS (mMRS) agar to determine the effect of sodium acetate on antifungal activity. The mMRS agar was prepared according to MRS (Oxoid) formula without sodium acetate (10 g/l peptone, 8g/l "Lab-Lemco" powder, 4g/l yeast extract, 20g/l glucose, 1ml/l $C_{24}H_{46}O_6$, 2g/l K_2HPO_4, 2g/l $C_6H_8O_7 \times 2NH_3$, 0.2g/l $MgSO_4 \times 7H_2O$ and 0.05g/l $MnSO_4 \times 4H_2O$).

2.4. Antifungal activity spectrum.

LAB isolates that possessed strong antifungal activity were screened against eight other moulds (*P. roqueforti*, *P. glabrum*, *P. solitum, G. candidum, A. versicolor, M. circinelloides, B. nivea, C. herbarum*) using the same overlay method described above with one LAB on each plate to determine their antifungal activity spectrum. These moulds were selected because of their common occurrence in cheese spoilage and ability to produce mycotoxins (*A. versicolor*).

2.5 Antifungal activity of LAB against P. commune *in cottage cheese.*

LAB that displayed strong antifungal activity on MRS agar were selected for testing against *P. commune* on cottage cheese. Some LAB with no antifungal activity were also selected as negative controls. LAB isolates were grown anaerobically in MRS broth for 48 hours at 30°C. After incubation, the optical density of each LAB in broth was measured and recorded, and 2ml was centrifuged at 16,873 x *g* (Eppendorf Centrifuge, Model 5418) for 1 minute to obtain the bacterial cell pellet. The spent MRS broth was discarded and bacterial cells were resuspended with peptone water (0.1% w/v) to obtain a standard number of cells per ml using the OD_{600nm} as a reference. Each LAB suspension was estimated to contain 1 x 10^9 CFU/ml and the exact concentration of each LAB suspension was determined by plating dilutions on MRS agar and incubation.

Twelve grams of commercially obtained cottage cheese which contained 11.3g protein, 5.4g fat and 2.6g sugar per 100g (Dairy Farmers, Queensland, Australia) were weighed out on each petri dish (9cm in diameter; Labtek) and inoculated with 0.1ml of LAB suspension. The cottage cheese was mixed on the petri dish using a sterile spreader for at least 1 minute to ensure even distribution of bacterial cells. Controls were prepared by inoculating cottage cheese with 0.1ml of peptone water not containing LAB. The plates were then incubated at room temperature (~24°C) for 2 days. *P. commune* fungal inoculum was prepared according to the method described earlier and 0.1ml (1 x 10^6 spores/ml) was inoculated onto each cottage cheese plate (final concentration was ~1 x 10^4 spores/g of cottage cheese), including control plates without LAB, and mixed again as above to ensure even distribution. Plates were incubated at room temperature and examined every few days for mould growth: (-) no mould growth; (+) small mould spots; (++) moderate sized mould spots or patches; (+++) mostly or completely covered by mould. Each strain was tested at least twice and consistent results were obtained. The data presented is from the replicate experiment which showed the least antifungal activity.

2.6. Identification of LAB.

LAB that possessed strong antifungal activity were selected for identification by sequence analysis of 16S rDNA (Ström, Sjögren, Broberg & Schnürer, 2002). The LAB were grown overnight in MRS broth anaerobically at 30°C and subsequently centrifuged to obtain the bacteria cell pellet. Bacteria DNA was extracted using the previously described method (Prasad & Turner, 2011). Amplification of the 16S rDNA gene was done by polymerase chain reaction (PCR) (94°C for 2 min, and 30 cycles of 94°C/20s, 53°C/30s, 72°C/1.5min) using primers 16S-S Forward (5'-AGAGTTTGATCCTGGCTC-3') and 16S-R Reverse (5'-CGGGAACGTATTCACCG-3'). The resulting PCR products were sent for purification and sequencing at Macrogen (South Korea). The partial 16S rDNA sequences of approximately 250-500bp were used to search public databases (Genbank using BLAST and the Ribosomal Database Project) for the identification of the LAB with the closest species match being reported.

3. Results

3.1. Screening of LAB for antifungal activity.

Out of the 897 LAB screened for antifungal activity against the indicator mould *P. commune*, approximately 7% showed antifungal activity during the initial screening. Figure 1 shows the percentage of antifungal LAB isolates out of the total number of LAB isolated from particular herbs, fruits and vegetables which were sources of antifungal LAB. A variety of sources yielded LAB with antifungal activity with thirty-six isolates showed weak inhibition (+), 11 isolates showed moderate inhibition (++), while 15 isolates showed strong (+++) inhibition (Figure 1). After re-testing, 12 out of 15 of the strong inhibiting isolates generated reproducible large zones of inhibition (Figure 2) and 4 isolates with no activity were also selected for further study as controls. LAB isolates that displayed antifungal activity on MRS agar did not produce the same antifungal activity when tested on modified MRS agar made without sodium acetate (data not shown).

3.2. Antifungal activity spectrum of LAB.

The 12 LAB selected after confirmation of antifungal activity on MRS were tested against a selection of moulds to determine the range of their inhibitory activity. Eight other mould species were chosen based on their involvement in cheese spoilage. It was found that all 12 isolates had the same inhibitory spectrum (activity against *P. solitum, A. versicolour* and *C. herbarum*, but not against *P. roqueforti, P. glabrum, G. candidum, M. circinelloides* and *B. nivea*).

3.3. Identification of anti-fungal LAB.

LAB species were identified via partial 16S rDNA sequencing (Table 1). Out of the 12 isolates that displayed antifungal activity, all isolates were identified as *Lactobacillus plantarum*. Four LAB that previously displayed no antifungal activity on MRS agar were identified as *Weissella soli*, *Lactococcus lactis*, *Leuconostoc inhae* and *Leuconostoc mesenteroides.*

3.4. Antifungal activity of LAB against P. commune *on cottage cheese.*

The results of the antifungal activity of different species of LAB against *P. commune* that was inoculated (~10^4 spores/g) on cottage cheese are shown in Table 1. The first control (control 1) was cheese that was inoculated with *P. commune* but without LAB, was found to display moderate mould growth from day 4 and was completely covered in dark green mould on day 12 (Figure 3). Cottage cheese inoculated with antifungal LAB *Lb plantarum* isolates did not show signs of mould growth until at least day 18 with some cheese not showing any visible mould at day 29 (Table 1 and Figure 3). Of note are two *Lb. plantarum* strains (#170 and 377) which were able to prevent visible mould growth beyond day 29, the last time-point of the experiment.

Four LAB isolates that did not display antifungal activity on MRS agars were also used as negative controls. Cheese containing *W. soli* #33, *Lc. lactis* #49, *Le. inhae* #402 and *Le. mesenteroides* #844 all had visible mould growth at day 4 and were completely covered in mould by day 12 (Table 1 and Figure 3).

4. Discussion

LAB have a long history of use in fermented food products and are generally regarded as safe organisms. Due to the production of lactic acid and several antimicrobial compounds, extensive studies have been conducted on their preservative potential, both against pathogenic bacteria (O'Sullivan, Ross & Hill, 2002) and fungi (Schnürer & Magnusson, 2005). Multiple publications have highlighted the ability of some LAB strains to repress mould growth (Dalié, Deschamps & Richard-Forgot, 2010) however, to the best of our knowledge, no studies were conducted on the antifungal activity of LAB isolated from a wide range of herbs, fruits and vegetables.

In the present study, 897 LAB previously isolated from herbs, fruits and vegetables were screened against an indicator mould *P. commune* on MRS agar using an overlay method to identify their antifungal properties. Twelve LAB isolates with strong or moderate antifungal activity were identified to species level and were all found to be *Lb. plantarum*. In our previous work, the same large collection of LAB isolates were tested for their antimicrobial activity against pathogens *Listeria monocytogenes* and *Salmonella* Typhimurium and the majority of the antimicrobial LAB were identified as species from *Lactococcus*, *Leuconostoc* and *Weissella,* including strains of *Le. mesenteroides, W. cibaria* and *Lc. lactis* (data not shown). Interestingly however, no *Lb. plantarum* were identified in this screening of LAB against *L. monocytogenes* and *S.* Typhimurium. This shows that the *Lb. plantarum* identified in this study may possess antifungal but not antibacterial activity and that the screening methods used are highly specific for the target organism i.e. mould. Likewise a recent similar large scale screening of around 7000 isolates of presumptive LAB from a variety of sources against *Penicillium expansum* identified *Lb. plantarum* strains as the most common antifungal LAB (Crowley, Mahony, & van Sinderen, 2013a). Results of our current study also showed that the antifungal activity of LAB is likely strain dependent. *Lb. plantarum* isolated from different sources displayed varying degrees of antifungal activity (e.g. isolates #170 and #892). This suggests that not all LAB of the same genera and species may be used as biopreservatives and further steps are likely needed to be taken to differentiate strains of bacteria using genotyping methods.

In order to investigate their effectiveness as cheese preservatives, these antifungal LAB were screened against a variety of moulds commonly associated with cheese to evaluate their antifungal activity spectrum. According to ICMSF (1998) and Taniwaki et al. (2001), commonly isolated spoilage fungi from cheese include *Penicillium, Aspergillus, Cladosporium, Geotrichum, Mucor and Trichoderma*, with *Penicillium* being the most predominant flora associated with cheese spoilage. Among the *Penicillium* sp., *P. commune* and *P. roqueforti,* both of which have been involved in cheese production, are the two most common spoilage species found in Australian and New Zealand retail cheeses (Hocking, 1994). Although the antifungal activity spectrum of the antifungal LAB identified in this study is not very wide, the selected LAB isolates were found to be inhibitory against several important spoilage moulds (*P. solitum, C. herbarum* and *A. versicolour)* in cheese. According to Oyugi and Buys (2007) and Hocking (1994), *P. solitum* is the main species of mould found in shredded cheeses in South Africa and Australia while *C. herbarum* is commonly associated with cheese with thread mould defects in Australian cheese factories (Hocking, 1994). *A. versicolour*, though not as commonly isolated from cheese spoilage, is an important mould that produces mycotoxins that could potentially harm consumers. None of the LAB were found to be inhibitory against *P. roqueforti*, which makes it applicable as a biopreservative on blue cheese as *P. roqueforti* is often inoculated as secondary starter to achieve desired properties in blue cheese. Similarly, antifungal LAB have also shown an activity spectrum which excluded *P. roqueforti* (Magnusson, Ström, Roos, Sjögren, & Schnürer, 2003).

The effect of sodium acetate on the antifungal activity of LAB was also investigated and results showed that antifungal LAB requires sodium acetate in the MRS medium to exhibit antifungal activity. This finding is consistent with that of Schillinger and Villarreal (2010), who studied the influence of medium composition on the antifungal activity of LAB and observed that LAB strains, previously shown to inhibit mould on MRS agar, were unable to produce inhibition zones when grown on MRS medium without sodium acetate or with reduced glucose content. The authors also observed antifungal activity only when normal MRS broth containing 61mmol/l sodium acetate was used. Similarly, Cabo et al. (2002) and Stiles et al. (2002) also reported a synergistic effect between acetate present in the growth medium with lactic acid and other antifungal compounds produced by the LAB, and is likely to be the main factor responsible for the antifungal properties of the selected strains. Our finding further confirms the role of sodium acetate on the antifungal activity of LAB and should be taken into consideration when evaluating the antifungal activity of LAB on MRS medium. However, it should be noted that the absence of sodium acetate from MRS agar did not affect the antifungal activity of propionibacteria on *P. roqueforti* and *Aspergillus fumigatus* (Lind, Jonsson & Schnürer, 2005).

In order to evaluate the potential of these antifungal LAB as food biopreservatives, we tested them against *P. commune* on cottage cheese. Few papers reported the use of antimicrobial LAB in cheese, especially for their use in preventing mould spoilage. Neugebauer and Gilliland (2005) studied the antagonistic action of *Lb. delbrueckii* RMZ-5 against *Pseudomonas fluorescens* on cottage cheese and found that the number of spoilage organisms did not increase over 21 days with a treatment of 1 x 10^9 CFU/g *Lb. delbrueckii* RMZ-5. Strains of *Bifidobacterium infantis* and *Bifidobacterium breve* were also found to reduce the levels of *Pseudomonas* on cottage cheese (O'Riordan & Fitzgerald, 1998). Along with these studies using cottage cheese as a model, we also found this product to be a quick and simple matrix for evaluation of the biopreservative potential of LAB on cheese. In our study, antifungal isolates of *Lb. plantarum*, were all able to prevent the growth of *P. commune* on cottage cheese for up to 14 to >25 days more than the no LAB control, however future work looking at testing these LAB in other cheese will be of interest.

The isolation of antifungal *Lb. plantarum* in this study supports the findings of several publications on the antifungal activity of this species. *Lb. plantarum* strains have been extensively investigated as mould controlling agents in bread where they have shown positive results (Coda et al., 2011; Dal Bello et al., 2007; Moore, Dal Bello and Arendt, 2008). The use of *Lb. plantarum* in combination with calcium propionate was also found to inhibit mould growth better than using calcium propionate alone (Ryan, Dal Bello & Arendt, 2008). Besides the application on baked products, Sathe, Nawani, Dhakephalkar and Kapadnis

(2007) studied the potential of LAB to prolong shelf life of fresh vegetables and found that cell-free supernatant of *Lb. plantarum* inoculated into vegetables were able to significantly delay fungal spoilage when challenged by *Aspergillus flavus, Fusarium graminearum, Rhizopus stolonifer* and *Botrytis cinerea.* Application in of *Lb. plantarum* to control mould in fruits, including apples and kumquats has also been demonstrated (Trias, Bañeras, Montesinos & Badosa, 2008; Wang, et al., 2013).

Besides lactic acid, several compounds have been identified as the mechanism of action of *Lb. plantarum*. Phenyllactic acid and two cyclic dipeptides cyclo (L-Leu-L-Pro) and cyclo (L-Phe-L-Pro) were identified in the cell-free supernatant of antifungal *Lb. plantarum* FST 1.7 (Dal Bello et al., 2007), while cyclic dipeptides cyclo(L-Phe–L-Pro) and cyclo(L-Phe–trans-4-OH-L-Pro), were identified as the mechanism of action of antifungal *Lb. plantarum* MiLAB393 (Ström, Sjögren, Broberg & Schnürer, 2002). Lavermicocca et al. (2000) also found that the production of phenyllactic and 4-hydroxy-phenyllactic acids contributed to the antifungal activity of *Lb. plantarum* 21B and in a later study, the same authors discovered that less than 7.5 mg/ml of phenyllactic acid were required to obtain full inhibition of mould (Lavermicocca, Valerio & Visconti, 2003). Niku-Paavola, Laitila, Mattila-Sandholm and Haikara (1999) also isolated and identified benzoic acid, methylhydantoin, mevalonolactone and cyclo (Gly-L-Leu) in the culture filtrate of *Lb. plantarum* VTT E-78076, and found it to be active against *Fusarium avenaceum* VTT D-80147. Recent work has identified antifungal active compounds from isolates of *Lb. plantarum* from kimchi as 5-oxododecanoic acid, 3-hydroxy decanoic acid and 3-hydroxy-5-dodecenoic acid (Ryu, Yang, Woo, & Chang, 2014) or 3,6-bis(2-methylpropyl)-2,5-piperazinedion (Yang & Chang, 2010). A genome shuffling approach has been used to enhance the antifungal activity of *Lb. plantarum* IMAU10014 (Wang, et al., 2013) and genome sequencing of antifungal *Lb. plantarum* strain 16 (Crowley, Bottacini, Mahony & van Sinderen, 2013) will provide a better understanding of the antifungal mechanisms of *Lb. plantarum*.

The current study shows that LAB from different fruits and vegetables and from different genera and species can exhibit antifungal activity against a number of common cheese spoilage moulds, particularly *P. commune.* The antifungal activity is observed not only on MRS agar, but on cottage cheese as well, indicating potential for the control of spoilage moulds in cheese products. Further investigations to identify the minimum inhibitory concentration (MIC) of each species, as well as the characterization of the LAB antifungal compounds may help in understanding the antifungal activity of these LAB. In order for LAB to be successfully applied as biopreservatives in cheese, more studies need to be done to explore the effectiveness on different variety of cheeses as well as the methods of application. Sensory analysis would also be required to determine whether the LAB and/or their antifungal compounds would impart undesirable flavours to the cheese and cheese products.

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Figure legends.

Figure 1. Sources of LAB isolates with different levels of antifungal activity against *P. commune* on MRS agar. Numbers on the bars indicate number of LAB in each group, while the percentage of isolates from each food source is shown on the X-axis. Note: only foods are shown which contained antifungal LAB. Antifungal activity scale (-, +, ++ and +++) is mentioned in section 2.2.

Figure 2. Antifungal activity of LAB isolates 49 (antifungal score of -), 897 and 892 (antifungal scores of +++) on MRS agar overlaid with *P. commune*. A and B are images from the bottom and top of the same agar plate, respectively.

Figure 3. Images showing growth of *P. commune* on cottage cheese with or without inoculated LAB.

Table 1. Antifungal activity of selected LAB against *P. commune* on cottage cheese.

No.	Species	Source	LAB CFU/g	***P. commune* growth on cottage cheese***								
				Day								
				0	**4**	**8**	**12**	**16**	**18**	**20**	**25**	**29**
LAB with antifungal activity on MRS agar												
170	*Lb. plantarum*	Stevia (sweet leaf)	10^7	-	-	-	-	-	-	-	-	-
377	*Lb. plantarum*	Baby endive	10^7	-	-	-	-	-	-	-	-	-
845	*Lb. plantarum*	Parsnips	10^7	-	-	-	-	-	-	+	+	+
880	*Lb. plantarum*	Asian Vegetables	10^8	-	-	-	-	-	+	+	+	+
883	*Lb. plantarum*	Spinach	10^7	-	-	-	-	-	-	-	+	+
884	*Lb. plantarum*	Cos Lettuce	10^7	-	-	-	-	-	+	+	++	++
885	*Lb. plantarum*	Broccoli	10^7	-	-	-	-	-	+	+	+	+
871	*Lb. plantarum*	Red capsicum	10^7	-	-	-	-	-	+	+	+	+
891	*Lb. plantarum*	Cos lettuce	10^7	-	-	-	-	-	+	+	+	++
892	*Lb. plantarum*	Broccoli	10^7	-	-	-	-	-	+	+	++	+++
895	*Lb. plantarum*	Spinach	10^7	-	-	-	-	-	+	+	+	+
897	*Lb. plantarum*	Green bean	10^7	-	-	-	-	-	-	+	+	+
Control LAB with no antifungal activity on MRS agar												
33	*W. soli*	Mixed salad	10^7	-	++	++	+++	+++	+++	+++	+++	+++
49	*Lc. lactis*	Flat parsley	10^7	-	+	+	+++	+++	+++	+++	+++	+++
402	*Le. inhae*	Baby Rocket	10^8	-	+	+++	+++	+++	+++	+++	+++	+++
844	*Le. mesenteroides*	Rocket leaves	10^7	-	+	+	+++	+++	+++	+++	+++	+++
Controls												
1	no LAB		0	-	++	+++	+++	+++	+++	+++	+++	+++
2	no LAB and no *P. commune*		0	-	-	-	-	-	-	-	-	-

*Unless stated all cheese samples were inoculated with *P. commune*. Scoring was as follows: (-) no mould growth; (+) small mould spots; (++) moderate sized mould spots or patches; (+++) mostly or completely covered by mould.

Figure 1.

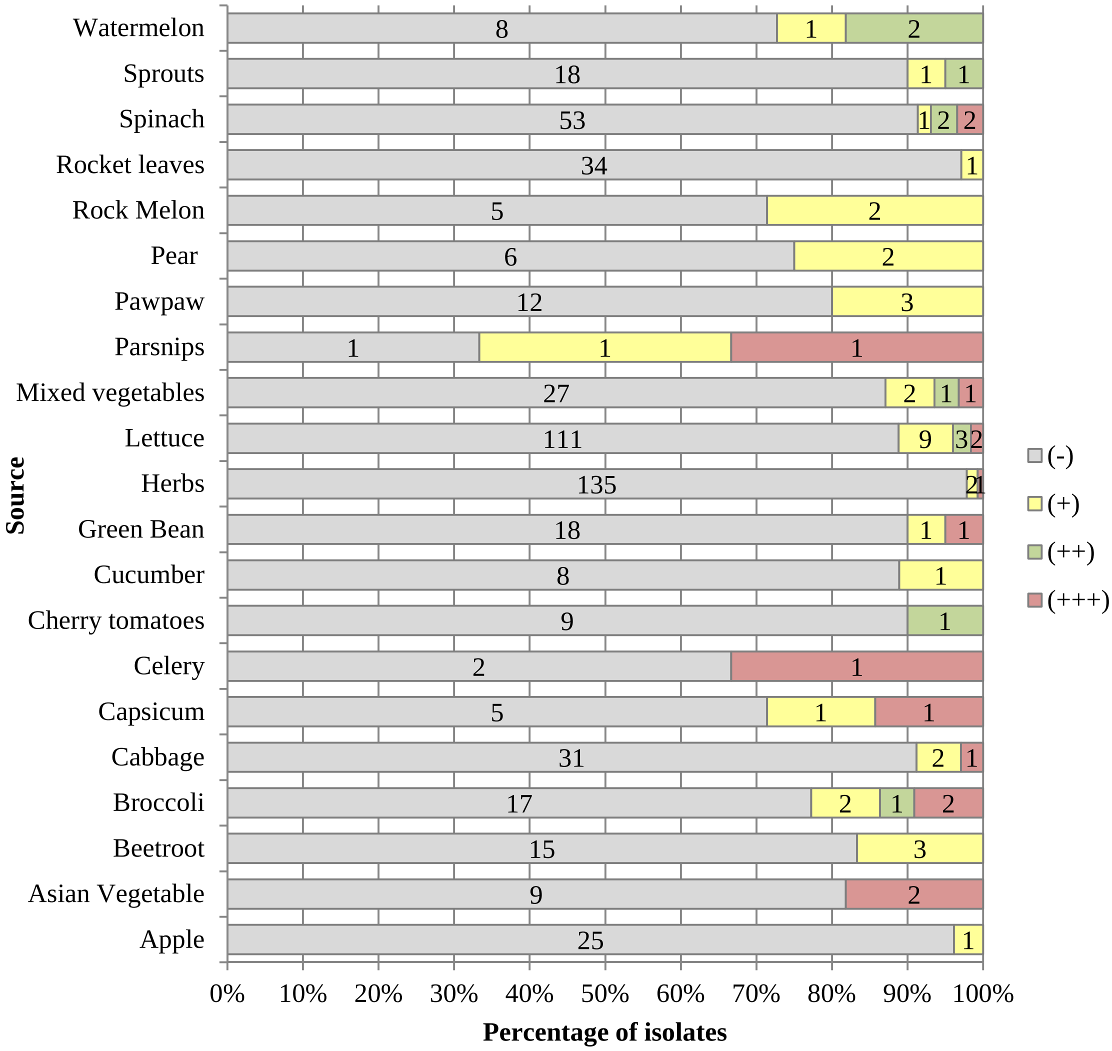

Watermelon
8
1
2
Sprouts
18
1
1
Spinach
53
1
2
2
Rocket leaves
34
1
Rock Melon
5
2
Pear
6
2
Pawpaw
12
3
Parsnips
1
1
1
Mixed vegetables
27
2
1
1
Lettuce
111
9
3
2
Herbs
135
2
1
Green Bean
18
1
1
Cucumber
8
1
Cherry tomatoes
9
1
Celery
2
1
Capsicum
5
1
1
Cabbage
31
2
1
Broccoli
17
2
1
2
Beetroot
15
3
Asian Vegetable
9
2
Apple
25
1
Source
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
Percentage of isolates
(-)
(+)
(++)
(+++)

Figure 2.

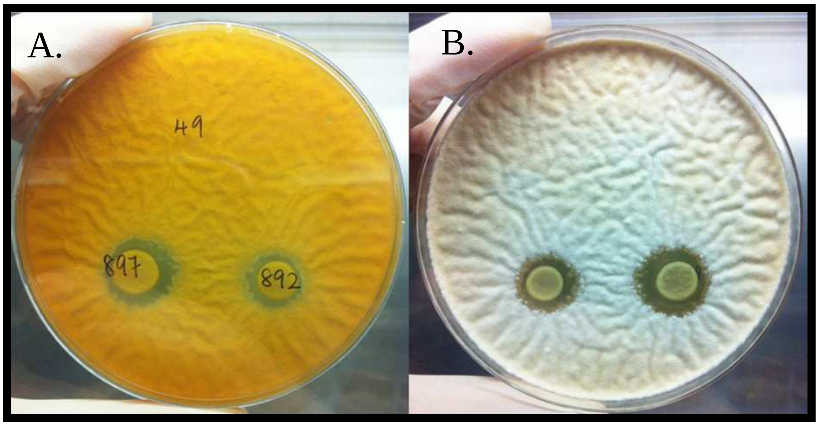
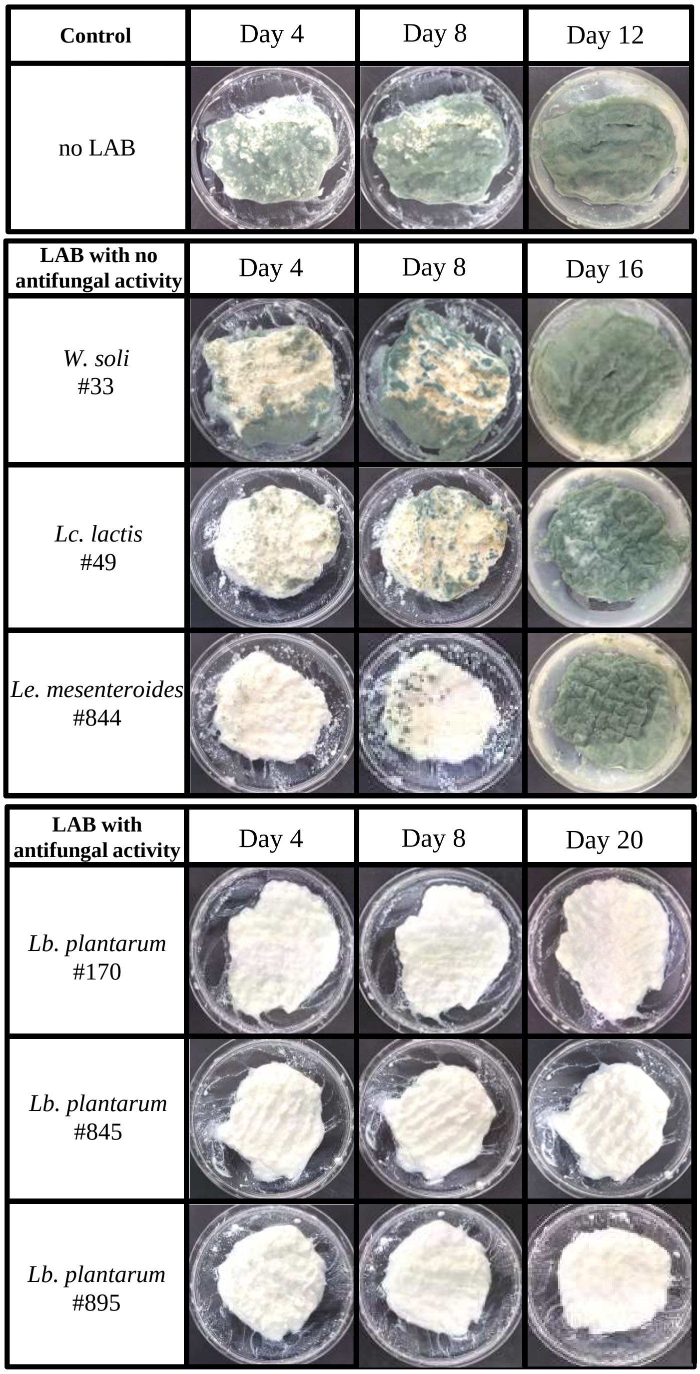


Figure 3.


Control
Day 4
Day 8
Day 12
no LAB
LAB with no antifungal activity
Day 4
Day 8
Day 16
W. soli #33
Lc. lactis #49
Le. mesenteroides #844
LAB with antifungal activity
Day 4
Day 8
Day 20
Lb. plantarum #170
Lb. plantarum #845
Lb. plantarum #895

Highlights

► Antifungal activity of 897 lactic acid bacteria was tested against *Penicilium commune*.

► All 12 strong antifungal lactic acid bacteria were *Lactobacillus plantarum*.

► Inhibition of *P. solitum, Aspergillus versicolour* & *Cladosporium herbarum* was seen.

► Antifungal isolates significantly inhibited *P. commune* growth in cottage cheese.

LAB strains which possessed different levels of antifungal activity (scored as +, ++ or +++) on MRS agar

Activity (+)

Strain No.	Source of LAB
46	Assorted lettuce (organic lettuce)
96	Cos lettuce
89	Fresh herb
127	Fancy lettuce
147	Beetroot
151	Cos lettuce
186	Green cabbage
212	Coriander
229	Chinese Broc
238	Baby leaves with beetroot
245	Traditional stir fry vegetables
276	Mixed salad: green lettuce, red lettuce, spinach, rocket lettuce.
280	Aromatic spinach blend
285	Baby leaves with beetroot
336	Baby red capsicums
356	Snow pea sprouts
365	Parsley
391	Summer lettuce
400	Summer lettuce
401	Baby rocket
499	Iceberg lettuce
513	Baby cos leaf
526	Iceberg lettuce
543	Cucumber
548	Nashi
552	Apple (Pink lady)
685	Chinese cabbage
735	Pawpaw
741	Persimmous pear
744	Rock melon
749	Rock melon
762	Watermelon
782	Pawpaw
789	Pawpaw
817	Green Bean
827	Parsnips

Activity (++)

Strain No.	Source of LAB
97	Cos Iceberg lettuce

99	Iceberg lettuce
181	Chinese Broc
217	Baby spinach
269	Traditional stir fry vegetables
275	Cherry tomatoes
289	English spinach
505	Iceberg lettuce
758	Watermelon
759	Watermelon
804	Organically Spouts

Activity (+++)

Strain No.	Source of LAB
845	Parsnips
883	Vietnamese spinach (mong toi)
884	Cos lettuce
885	Brocolli
890	Asian vegetable (Cai ngot)
891	Cos lettuce
897	Green Bean
895	Vietnamese spinach (mong toi)
170	Sweet leaves
871	Capsicum red
880	Asian Vegetable (Rau den)
892	Brocolli

List of LAB screened for antifungal activity and their source

Strain No.
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41

Source of LAB
Mesculin leaves (salad mix)
Coriander
Salad mix
Midi cos
Mesculin leaves (salad mix)
Rocket leaves
Green oak lettuce
Coriander
Coriander
Salad mix
Spinach leaves
Green oak lettuce
Rocket leaves
Rocket leaves
Mesculin leaves (salad mix)
Flat parsley
Baby spinach
Red and green lettuce
Baby spinach
Iceberg lettuce
Fancy lettuce
Mixed salad: green lettuce, red lettuce, spinach, rocket lettuce.
Green lettuce
Iceberg lettuce
Red and green lettuce
Baby spinach
Mixed salad: green lettuce, red lettuce, spinach, rocket lettuce.
Baby spinach
Mixed salad: green lettuce, red lettuce, spinach, rocket lettuce.
Thai basil
Thai basil
Parley
Mixed salad: green lettuce, red lettuce, spinach, rocket lettuce.
Parley
Thai basil
Cos lettuce
Cos lettuce
Baby spinach
Baby spinach
Parley
Basil

Mixed salad: green lettuce, red lettuce, spinach, rocket lettuce.
Flat parley
Cos lettuce
Iceberg lettuce
Assorted lettuce (organic lettuce)
Iceberg lettuce
Iceberg lettuce
Flat parley
Flat parley
Assorted lettuce (organic lettuce)
Mixed salad: green lettuce, red lettuce, spinach, rocket lettuce.

Fancy lettuce
Parley
Iceberg lettuce
Baby spinach
Iceberg lettuce
Mixed salad: green lettuce, red lettuce, spinach, rocket lettuce.
Basil
Thai basil
Flat parley
Mixed salad: green lettuce, red lettuce, spinach, rocket lettuce.
Parley
Parley
Sweet basil
Parley
Baby spinach
Sweet basil
Parley
English spinach
Coriander
Coriander
Coriander
Assorted lettuce (organic lettuce)
Red and green lettuce
English spinach
Iceberg lettuce
Parley
Fancy lettuce
Coriander
Cos lettuce
Coriander
English spinach
Baby spinach
Coriander
Red and green lettuce
Fancy lettuce
Celery
Fresh herb
Coriander

Parley
Parley
Fancy lettuce
Coriander
Celery
Cos lettuce
Cos Iceberg lettuce
Celery
Iceberg lettuce
Cos lettuce
Coriander
Parley
Red and green lettuce
Celery
Celery
Iceberg lettuce
Cos lettuce
Coriander
Iceberg lettuce
Iceberg lettuce
Coriander
Iceberg lettuce
Cos Iceberg lettuce
Cos Iceberg lettuce
Cos Iceberg lettuce
Fancy lettuce
Red and green lettuce
Celery
Fancy lettuce
Fancy lettuce
Fancy lettuce
Iceberg lettuce
Cos Iceberg lettuce
Fancy lettuce
Fancy lettuce
Iceberg lettuce
Fancy lettuce
Iceberg lettuce
Parley
Iceberg lettuce
Spinach leaves
Cos lettuce
Mixed salad: green lettuce, red lettuce, spinach, rocket lettuce.
Fancy lettuce
Mixed salad: green lettuce, red lettuce, spinach, rocket lettuce.
Fresh herb
Parley
Organically sprouted
Fresh herb
sweet potato leaves

Red and green lettuce
Green bean leaves
Fresh herb
Mint
Mint
Beetroot
Beetroot
Beetroot
Fresh herb
Beetroot
Cos lettuce
Chinese cabbage
Cos lettuce
Mint
Pumpkin leaves
sweet potato leaves
Chinese mint
Chinese cabbage
Coriander
Mint
Chinese mint
Fancy lettuce
Chinese cabbage
Coriander
Sweet leaves
Pumpkin leaves
Coriander
Chinese mint
Chinese mint
Sweet leaves
sweet potato leaves
Chinese mint
sweet leaves
Pumpkin leaves
Green cabbage
Green cabbage
Coriander
Baby Choy sum
Green cabbage
Pak choy
Chinese Broc
Celery
Broccoli
Celery
Broccoli
Green cabbage
Celery
Iceberg lettuce
Pak choy
Green bean leaves

sweet potato leaves
Pumpkin leaves
Pumpkin leaves
sweet potato leaves
Mint
Baby Choy sum
Celery
Coriander
Celery
Baby Choy sum
Fancy lettuce
Pumpkin leaves
Broccoli
Beetroot
Mint
Chinese Broc
Celery
Sweet leaves
Beetroot
Mint
Fresh herb
Coriander
Beetroot
Pumpkin leaves
Pumpkin leaves
Beetroot
Baby spinach
Iceberg lettuce
Celery
Mint
Mint
Baby spinach
Celery
Celery
Green bean leaves
Fresh herb
Mint
Broccoli
Chinese Broc
Green bean leaves
Fresh herb
Salad mix
Baby spinach
Fancy lettuce
Fresh herb
Mint
Fresh herb
Baby leaves with beetroot
Baby Asian greens
Aromatic spinach blend

Aromatic spinach blend
Rainbow salad
Just broccoli
Traditional stir fry vegetables
Traditional stir fry vegetables
Thyme
Thyme
Baby Asian greens
Thyme
Snow pea sprouts
Baby brussels sprouts
Baby brussels sprouts
Coleslaw
Coleslaw
Cherry tomatoes
Baby brussels sprouts
Aromatic spinach blend
Rainbow salad
Aromatic spinach blend
Baby leaves with beetroot
Baby rocket
Tuscan cabbage
Aromatic spinach blend
Baby leaves with beetroot
Mint
Baby red capsicums
Coleslaw
Rainbow salad
Traditional stir fry vegetables
Rainbow salad
Spinach, rocket and kale
Baby brussels sprouts
Rainbow salad
Rainbow salad
Cherry tomatoes
Mixed salad: green lettuce, red lettuce, spinach, rocket lettuce.
Coleslaw
Baby Asian greens
Mint
Aromatic spinach blend
Baby rocket
Aromatic spinach blend
Traditional stir fry vegetables
Mint
Baby leaves with beetroot
Baby leaves with beetroot
Baby brussels sprouts
Baby brussels sprouts
English spinach
Iceberg lettuce

Baby leaves with beetroot
Traditional stir fry vegetables
Traditional stir fry vegetables
Rocket salad mix
Rocket salad mix
Cherry tomatoes
Cherry tomatoes
English spinach
Spinach leaves
English spinach
Continental parley
Coleslaw
Tuscan cabbage
Salanova red coral lettuce
Sweet raspberries
Spinach leaves
Rocket salad mix
Baby Endive
Mixed salad: green lettuce, red lettuce, spinach, rocket lettuce.
Tuscan cabbage
Iceberg lettuce
English spinach
Mint
Spinach leaves
Mint
English spinach
Strawberries
Butter lettuce
Tarragon leaves
Oregano
English spinach
Rocket salad mix
Baby Asian greens
Rocket leaves
Strawberries
Iceberg lettuce
Tuscan cabbage
English spinach
Spinach leaves
English spinach
Baby Endive
Mint
Strawberries
Spinach leaves
Oz Berries
Baby red capsicums
Strawberries
Oregano
Coleslaw
Tuscan cabbage

Snow pea sprouts
Rocket leaves
Tuscan cabbage
Thyme
Treviso radicchio
Baby red capsicums
Tarragon leaves
Treviso radicchio
Baby Endive
Butter lettuce
Iceberg lettuce
Sweet raspberries
Thyme
Treviso radicchio
Baby red capsicums
Snow pea sprouts
Mixed salad: green lettuce, red lettuce, spinach, rocket lettuce.
Salanova red coral lettuce
Thyme
Thyme
Rocket leaves
Tuscan cabbage
Treviso radicchio
Cherry tomatoes
Parsley
Sweet raspberries
Treviso radicchio
Bistro salad
Tarragon leaves
Butter lettuce
Sweet raspberries
Sweet raspberries
Oz Berries
English spinach
Butter lettuce
Treviso radicchio
Baby Endive
Thyme
Baby rocket
Oregano
Tarragon leaves
Baby red capsicums
Iceberg lettuce
Four leaf salad mix
Spinach leaves
Wild rocket
Spinach leaves
Baby mesculin
Summer lettuce
Baby mesculin

Summer lettuce
Summer lettuce
Spinach leaves
Fresh Italian Parsley
Fresh Italian Parsley
Baby rocket
Spinach leaves
Basil
Summer lettuce
Summer lettuce
Baby rocket
Baby rocket
Summer lettuce
Spinach leaves
Spinach leaves
Coriander
Spinach leaves
Spinach leaves
baby mescullin salad
Baby spinach
Baby spinach
Baby spinach
Red oak lettuce
Fresh Italian Parsley
Fresh Italian Parsley
Wild rocket
Baby spinach
Four leaf salad mix
Baby spinach
Wild rocket
Baby spinach
Rocket leaves
Rocket leaves
Leafy mix
Leafy mix
Green oak lettuce
Leafy mix
Leafy mix
Leafy mix
Red oak lettuce
Red oak lettuce
Red oak lettuce
Red oak lettuce
Butter lettuce
Baby mesculin salad
Summer lettuce
Baby rocket
Red oak lettuce
Baby mesculin salad
Summer lettuce

Wild rocket
Baby mesculin salad
Four leaf salad mix
Baby spinach
Rocket leaves
Butter lettuce
Mint leaves
Baby rocket
Parsley
Mint leaves
Coriander
Baby rocket
Coriander
Mint leaves
Leafy mix
Mint leaves
Baby coslettuce
Coriander
Mint leaves
Fresh Italian Parsley
Red oak lettuce
Basil
Rosemary
Basil
Rocket leaves
Rocket leaves
Fresh Italian Parsley
Basil
Rocket leaves
Baby coslettuce
Parsley
Green oak lettuce
Basil
Parsley
Coriander
Parsley
Coriander
Red oak lettuce
Leafy mix
Mint leaves
Mint leaves
Parsley
Butter lettuce
Green oak lettuce
Parsley
Parsley
Rosemary
Rocket leaves
Leafy mix
Mint leaves

Basil
Mixed florets (Broccoli)
Baby cos leaf
Gourmet salad
Mixed florets (Broccoli)
Fancy lettuce
Iceberg lettuce
Gourmet salad
Iceberg lettuce
Mixed florets (Broccoli)
Celery
Iceberg lettuce
Fancy lettuce
Gourmet salad
Iceberg lettuce
Celery
Gourmet salad
Mixed florets (Broccoli)
Fancy lettuce
Baby cos leaf
Mixed florets (Broccoli)
Gourmet salad
Baby cos leaf
Gourmet salad
Fancy lettuce
Iceberg lettuce
Fancy lettuce
Gourmet salad
Baby cos leaf
Iceberg lettuce
Mixed florets (Broccoli)
Baby cos leaf
Celery
Gourmet salad
Baby cos leaf
Iceberg lettuce
Baby rocket
Baby rocket
Baby rocket
Coriander
Fresh herb
Fresh herb
Onion
Eggplant
Mint
Green bean
Fresh herb
Cucumber
Eggplant
Onion

Dragon bean
Cucumber
Cucumber
Dragon bean
Mint
Eggplant
Tomato
Nashi
Nashi
Nashi
Eggplant
Apple (Pink lady)
Dragon bean
Green bean
Green bean
Onion
Nashi
Roma tomato
Onion
Fresh herb
Tomato
Onion
Green bean
Apple (Pink lady)
Green bean
Green bean
Onion
Mint
Dragon bean
Nashi
Apple (Pink lady)
Fresh herb
Cucumber
Fresh herb
Nashi
Green bean
Dragon bean
Green bean
Dragon bean
Roma tomato
Green bean
Roma tomato
Tomato
Nashi
Green bean
Tomato
Cucumber
Tomato
Onion
Dragon bean

Green bean
Onion
Dragon bean
Green bean
Zaccuchi
Nashi
Red grape
Cherry tomatoes
Banana
Nashi
Nashi
Tomato
Mint
Zaccuchi
Zaccuchi
Zaccuchi
Green bean
Cherry tomatoes
Banana
Zaccuchi
Red grape
Cherry tomatoes
Cherry tomatoes
Roma tomato
Starfruit
Nashi
Cherry tomatoes
Tomato
Endive
Basil
Mint
Red grape
Purple perilla
Purple perilla
Strawberries
Strawberries
Fuji apple
Red grape
Fuji apple
Eggplant
Starfruit
Starfruit
Banana
Pawpaw
Fuji apple
Strawberries
Pawpaw
Tomato
Fresh herb
Apple (Granny Smith)

Apple (Granny Smith)
Red grape
Apple (Granny Smith)
Mint
Tomato
Starfruit
Fuji apple
Apple (Granny Smith)
Cucumber
Parsley
Fenugreek
Red mustard
Purple perilla
Starfruit
Red grape
Apple (Granny Smith)
Starfruit
Red grape
Cucumber
Apple (Pink lady)
Apple (Granny Smith)
Cucumber
Red grape
Kiwi
Royal gala (apple)
Kiwi
Chinese cabbage
Kiwi
Pineapple
Susu
Roma tomato
Pineapple
Pineapple
Royal gala (apple)
Susu
Kiwi
Chinese cabbage
Pineapple
Green chilli
Chinese cabbage
Chinese cabbage
Kiwi
Kiwi
Green chilli
Chinese cabbage
Royal gala (apple)
Kiwi
Chinese cabbage
Susu
Green chilli

Green chilli
Kiwi
Sugar loaf cabbage
Susu
Royal gala (apple)
Chinese cabbage
Royal gala (apple)
Susu
Susu
Kiwi
Royal gala (apple)
Kiwi
Royal gala (apple)
Kiwi
Kiwi
Susu
Chinese cabbage
Kiwi
Green chilli
Susu
Royal gala (apple)
Kiwi
Sugar loaf cabbage
Sugar loaf cabbage
Sugar loaf cabbage
Sugar loaf cabbage
Sugar loaf cabbage
Pawpaw
Pawpaw
Pawpaw
Mint
Mint
Spearmint
Fenugreek
Mint
Chervil
Endive
Endive
Endive
Fenugreek
Parsley
Parsley
Honeydew melon
Persimmous pear
Pawpaw
Pawpaw
Rock melon
Rock melon
Watermelon
Honeydew melon

Persimmous pear
Honeydew melon
Watermelon
Rock melon
Honeydew melon
Honeydew melon
Persimmous pear
Pawpaw
Rock melon
Rock melon
Watermelon
Watermelon
Rock melon
Watermelon
Persimmous pear
Rock melon
Watermelon
Watermelon
Watermelon
Watermelon
Honeydew melon
Watermelon
Chives
Chervil
Basil
Spearmint
Watermelon
Bosc pear
Red delicious apple
Spearmint
Fuji apple
Pawpaw
Pear
Pawpaw
Pink lady apple
Pawpaw
Pink lady apple
Pink lady apple
Pawpaw
Sugar loaf cabbage
Sugar loaf cabbage
Pawpaw
Sugar loaf cabbage
Sugar loaf cabbage
Tomatoes Gourmet
Witlof
Witlof
Tomatoes Gourmet
Pawpaw
Witlof

Organically Spouts
Witlof
Brussel Sprouts Loose
Brussel Sprouts Loose
Choy sum
Swiss Brown Mushrooms
Swiss Brown Mushrooms
Choy sum
Tomatoes Gourmet
Brussel Sprouts Loose
Swiss Brown Mushrooms
Pawpaw
Bosc Pear
Organically Spouts
Brussel Sprouts Loose
Tomatoes Gourmet
Coriander
Mushrooms Button
Mushrooms Flat
Mushrooms Button
Strawberries
Italian Parsley
Chives
Bean Sprouts
Okara
Asian Mix
Green Bean
String Bean
Baby Brocolli
Sugarsnap Peas
Turinps
Beetroot
Organically Spouted
Beetroot
Organically Sprouted
Zucchini
Parsnips
Yellow squash
Yellow squash
Organically Sprouted
Potatoes
Zucchini
Snow pea
Organically Sprouted
Organically Sprouted
Potatoes
Organically Sprouted
Parsnips
Kipfler Potatoes
Radish Bunch

Snow pea
Potatoes
Baby spinach
Rocket Leaves
Parsnips
Mesculin Leaves
Snow pea
Radish Bunch
Rocket Leaves
Kipfler Potatoes
Baby spinach
Tomatoes Gourmet
Tomatoes Brushed
Zucchini Green
Mangoes
Cabbage
Beetroot
Kiwifruit Green
Potatoes Kipfler
Zucchini Green
Tomatoes Brushed
Capsicum red
Chokoe
Mangoes
Pears
Zucchini Green
Cucumber Lebanese
Potatoes Kipfler
Tomatoes Gourmet
Cabbage
Capsicum red
Asian Vegetable (Rau den)
Green Bean
Chieves
Banana (lady finger)
Eggplant
Eggplant
Nectarine Yellow
Asian Vegetable (Cai ro)
Asian Vegetable (Rau den)
Chieves
Asian Vegetable (Rau den)
Vietnamese spinach (mong toi)
Cos lettuce
Brocolli
Glory Morning
Beetroot
Asian vegetable (Cai ngot)
Mint Leaves
Asian vegetable (Cai ngot)

Cos lettuce
Brocolli
Banana (lady finger)
Glory Morning
Vietnamese spinach (mong toi)
Brocolli
Green Bean