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Firmicutes dominate the bacterial taxa within sugar-cane processing plants

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Sugar cane processing sites are characterised by high sugar/hemicellulose levels, available moisture and warm conditions, and are relatively unexplored unique microbial environments. The PhyloChip microarray was used to investigate bacterial diversity and community composition in three Australian sugar cane processing plants. These ecosystems were highly complex and dominated by four main Phyla, *Firmicutes* (the most dominant), followed by *Proteobacteria*, *Bacteroidetes*, and *Chloroflexi*. Significant variation ($p < 0.05$) in community structure occurred between samples collected from ‘floor dump sediment’, ‘cooling tower water’, and ‘bagasse leachate’. Many bacterial Classes contributed to these differences, however most were of low numerical abundance. Separation in community composition was also linked to Classes of *Firmicutes*, particularly *Bacillales*, *Lactobacillales* and *Clostridiales*, whose dominance is likely to be linked to their physiology as ‘lactic acid bacteria’, capable of fermenting the sugars present. This process may help displace other bacterial taxa, providing a competitive advantage for *Firmicutes* bacteria.

Sugar cane (*Saccharum officinarum* L.) is a perennial grass grown in many tropical countries. Globally, it is one of the most important staple crops, both in terms of total production (ranked #1 at 1,685 Million tonnes) and area planted (#13 at 23.8 Million Ha) (2010 data; <http://faostat.fao.org>). In many tropical countries, sugar cane production represents the most important land-use and agricultural commodity. The processing of sugar cane involves crushing of the cane stalks, with water added to enhance recovery of juices. The juices are then processed through to a clarified solution which is concentrated to syrup by evaporators. The syrup may be later crystallised to form solid sugar. A by-product of the process is bagasse, the fibrous residue of the cane stalk. This may itself be used in the sugar-cane processing, either to remove sediment from the juice clarification process, and/or burned to provide energy to operate the facility.

Sugar cane production sites offer an unusual habitat for microbial life. The inherent nature of sugar cane processing results in the accumulation of high levels of organic material, some of which contain a high percentage of labile C (sugars) and the rest of which is largely a mix of complex carbohydrates (bagasse/trash)¹. The sites themselves are located in tropical to sub-tropical environments (alongside cane farms) and, as such, are warm and humid. Moisture availability is enhanced by the high use of water throughout the processing system.

A diverse range of ‘micro niches’ can be identified as common to most processing sites. However, most microbiological investigations have focussed on microbiota affecting the quality of the sugar product, or those which affect industrial operation of the plant. In the first instance, fungi such as *Aspergillus*, *Penicillium*, *Saccharomyces*, *Torula*, *Pichia*, and bacteria including *Streptomyces*, *Pseudomonas*, and *Bacillus* are commonly reported as responsible for the deterioration of sugar^{2,3}. Cooling tower water (CTW) systems are often colonised by oligotrophic, thermophilic microbial communities with *Caulobacteriaceae*, *Sphingomonadaceae*, and *Microbacteriaceae* bacteria being the most abundant^{4,5}. These bacteria can form biofilms which reduce the efficiency of the cooling towers and affect production at the cane-mills.

Furthermore, microbial micro-niches result from the bagasse processing. Typically, bagasse is stock-piled (“heaped”) and rainwater is passed through the piles to minimize the risk of spontaneous combustion and the associated generation of contaminated water and is captured in floor dump sumps (FDS) before being further processed. If contaminated storm water, leachate and windborne bagasse enters a waterway it has the potential to reduce dissolved oxygen levels and in extreme circumstances this can result in contaminated water with possible negative outcomes such as fish kills¹. In comparison to the CTW, the bagasse leachate (BGL) represents a habitat highly enriched in nutrients (hemicellulose and others) but otherwise under a similar range of environmental conditions. In comparison to CTW, the microbial community with FDS is less well understood, yet also has



Table 1 | Richness (Margalefs index; d) of bacterial communities present at sugar-cane processing sites

Treatment	BGL	FDS	CTW	FDS	CTW	FDS	CTW
Location	Mackay		Maryborough		Prosperine		
Phylum	1.91	1.88	1.85	1.88	1.82	1.86	1.85
Class	3.81	3.77	3.70	3.75	3.64	3.72	3.70
Order	7.28	7.19	7.07	7.17	6.96	7.10	7.06
Family	13.47	13.29	13.07	13.25	12.86	13.13	13.06
Sub-family	16.41	16.20	15.93	16.15	15.68	16.01	15.92
OTU	78.94	77.94	76.64	77.69	75.41	76.99	76.59

important applied aspects relating to environmental (odor) and human health issues (exposure to microbial rich environments)¹.

The primary objective of this study was to compare microbial diversity within hemi-cellulose enriched (FDS) and non-hemicellulose enriched habitats (CTW) in a tropical environment. As a secondary goal, we further aimed to understand the diversity and structures of microbial communities within the processing plants of one of the most important global commodities - sugar cane.

Results

Alpha diversity. Across all the samples, 34 different Phyla were found to be present (Table 1), which represents approximately half of those detectable by PhyloChip. The total OUT's present was 1367 - approximately 16% of those covered on the array. A table summarising the number of taxa present for each sample is given in the Supplementary Information. Richness of microbial communities was assessed using Margalefs index (d). Not surprisingly, richness increased with taxonomic level across all samples (Table 1). The levels of richness in the bacterial communities were similarly high across all sites and for all micro-niches examined. No clear impacts of sample type or location were evident.

Similarly, the evenness of the bacterial communities was similar between sampling locations and micro-niches examined (Table 2). As such, the communities were not dominated by a few taxa and this evenness is balanced across the samples. Combined analysis of the data set (Tables 1 and 2) showed there was a high degree of correlation between richness and evenness in the samples (Spearman correlation $\rho = 0.926$; $p < 0.001$). Samples with high richness of taxa had greater evenness; were less inclined to be dominated by any particular taxa.

Identification of dominant taxa. The 'top100' probe intensity score data was used to describe the dominant bacterial community of the sugar-cane processing mill. At Phylum level, the bacterial communities for all samples were dominated by *Firmicutes*, *Proteobacteria*, *Bacteroidetes* and *Chloroflexi* (Figure 1). However, even when looking within the top100 intensity data, a large range of phyla (18 of the 34 in total detected by PhyloChip) were present (Figure 1). These included a range of common (e.g. Cyanobacteria,

Table 2 | Evenness (Pielous index; J') of bacterial communities isolated from sugar-cane processing sites

Treatment	BGL	FDS	CTW	FDS	CTW	FDS	CTW
Location	Mackay		Maryborough		Prosperine		
Phylum	0.464	0.519	0.474	0.502	0.486	0.475	0.526
Class	0.658	0.672	0.677	0.679	0.680	0.678	0.699
Order	0.814	0.764	0.821	0.793	0.803	0.828	0.785
Family	0.867	0.838	0.867	0.856	0.855	0.873	0.854
Sub-family	0.856	0.828	0.858	0.846	0.845	0.865	0.844
OTU	0.986	0.959	0.990	0.976	0.981	0.967	0.962

Acidobacteria, Chlorobi) and rarer (e.g. OP3, TM7, Natronoanaerobium) taxa.

At the Phylum, a similar pattern of abundance occurred for the dominant Phyla for each of the sugar-cane communities. Of near-equal importance were always *Firmicutes* and *Proteobacteria*; *Bacteroidetes* were always third most abundant; *Chloroflexi* always fourth (Figure 1). The 'minor placements' were distributed reasonably across the remaining Phyla (Figure 1).

Analysis of the same data but at the Class level provides deeper information as to the composition of the dominant fraction of the bacterial communities. Within the *Proteobacteria*, the dominant classes were α -, β -*Proteobacteria*, followed by γ -, and ϵ -*Proteobacteria* (Figure 2). δ -*Proteobacteria* were present but at relatively lower

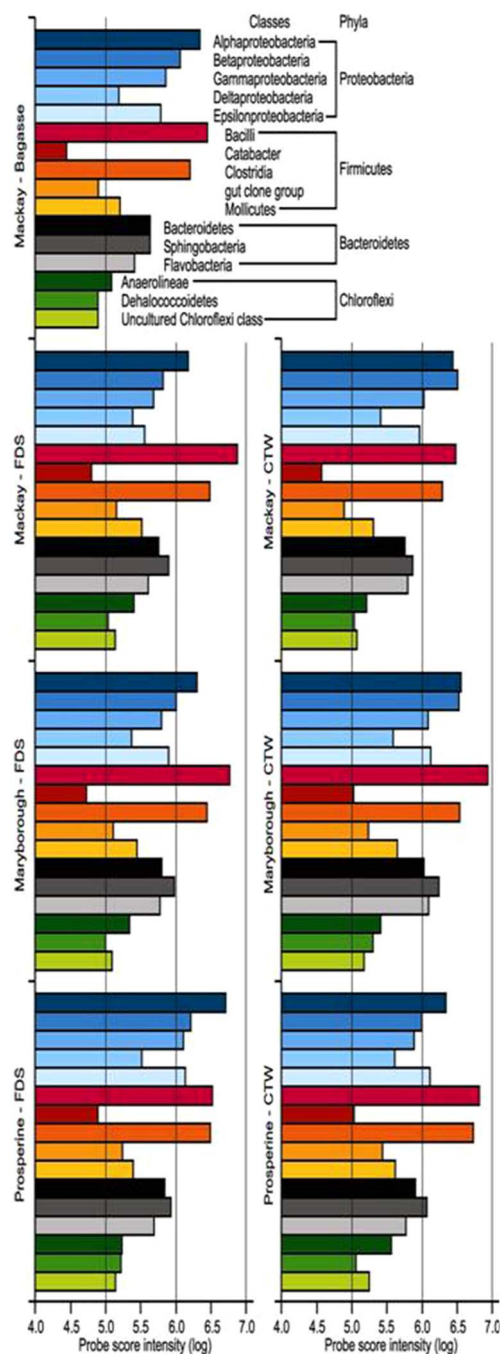


Figure 1 | Abundance of bacterial Phyla in samples of bagasse, floor dump sediment (FDS) and cooling tower water (CTW) from sugar-cane processing sites.

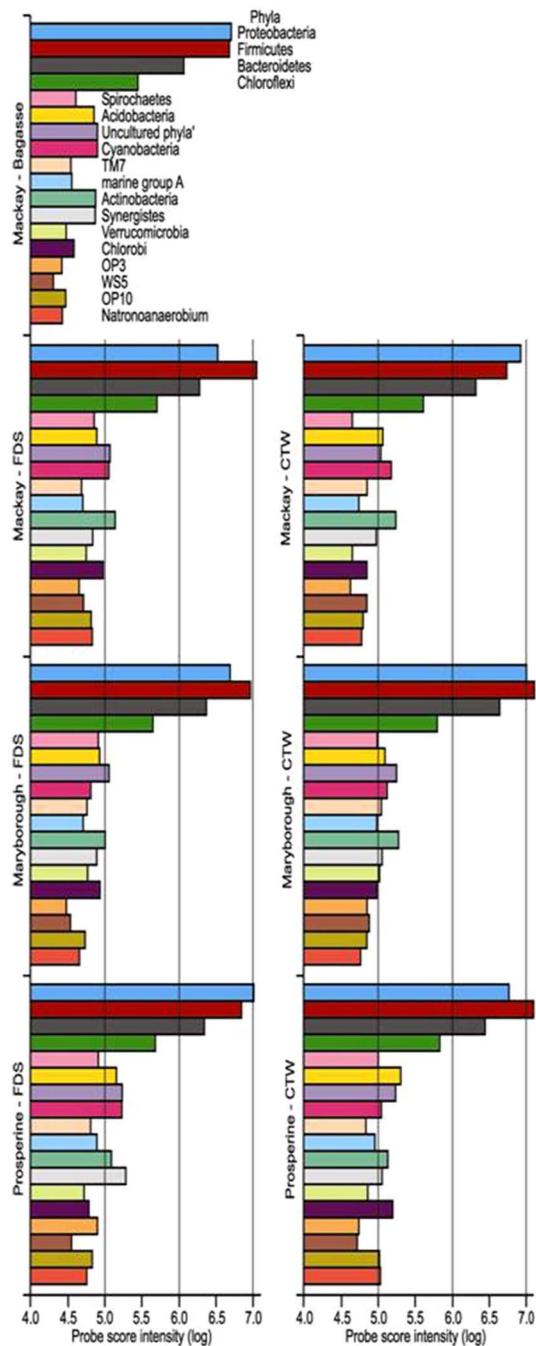


Figure 2 | Abundance of bacterial Classes in samples of bagasse, floor dump sediment (FDS) and cooling tower water (CTW) from sugar-cane processing sites.

abundance when compared to the other classes. The *Firmicutes* component of the community was dominated by Bacilli and Clostridia. In fact, Bacilli were the most dominant bacterial Class present in all but one sample (FDS at Proserpine; Figure 2). The Phylum *Bacteroidetes* were dominated by Classes Sphingobacteria and Bacteroidetes. Many other classes were present but contributed only a minor component of the overall community composition (Figure 2).

Effects of treatment (niches) and sampling location on community composition. The effect of treatment type and sampling location on bacterial community was tested using the whole PhyloChip data set. CAP analysis could not partition effects of 'location' over any of the taxonomic levels ($p > 0.05$; Table 2); i.e. the *Firmicutes*-dominated community type was consistent across all

Table 3 | Summary CAP analysis results testing for effects of treatment type (bagasse, FDS, and CTW) or location (Mackay, Maryborough, Proserpine) on bacterial community composition

	Treatment	Location
Phyla	0.019	0.783
Class	0.019	0.784
Order	0.095	0.816
Family	0.168	0.674
Sub-family	0.168	0.637
OTU	0.133	0.488

sugar-cane processing mills examined. However, significant effects of 'treatment' or niches/habitats were evident at high-taxonomic levels, with Phyla > Class > Order (Table 3). For all three taxa levels, the bagasse sample always partitioned from CTW or FDS samples. However, single allocation errors were made between one of the CTW and FDS samples (they were more similar to each other than their respective treatment groups).

Similarly, ordination by nMDS was used to visualise similarity in community composition between samples (at Class level). The bagasse sample was quite different to the FDS and CTW samples; however there is only a single observation for this treatment. A reasonable level of separation exists between the samples from CTW and FDS (Figure 4). Overall, the bacterial communities changed from bagasse-type, to FDS-type and then to CTW-type structures (Figure 3).

SIMPER analysis was used to determine which Classes of bacteria contributed towards defining differences in bacterial communities between the CTW and FDS treatments (Table 4). Most of the taxa contributing towards differences between the treatment groups were of minor relative abundance; as such, the rare bacterial groups were (generally) important in separating the two habitat types. A notable exception was the *Firmicutes*. Abundances of the Classes Bacillus, Lactobacillales, gut clone group, and Clostridia were all important in separating FDS and CTW communities; in all cases *Firmicutes* were of greater abundance in the FDS.

Discussion

Using high-density microarray tools (PhyloChip) a substantial level of bacterial diversity was uncovered in sugar-cane processing plants,

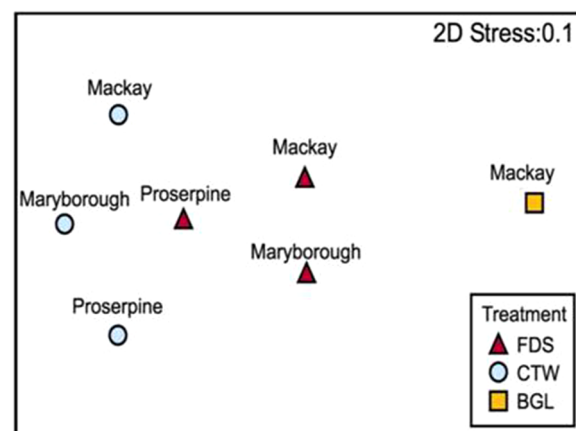


Figure 3 | Non-metric multi-dimensional scaling ordination (NMDS) plot showing similarity in community composition between samples. On the plot, increasing distance between sample points equates to more dissimilarity in community composition. Data is Class-level taxa and has been $\log(x + 1)$ transformed. Similarity is based on Gower's similarity method.

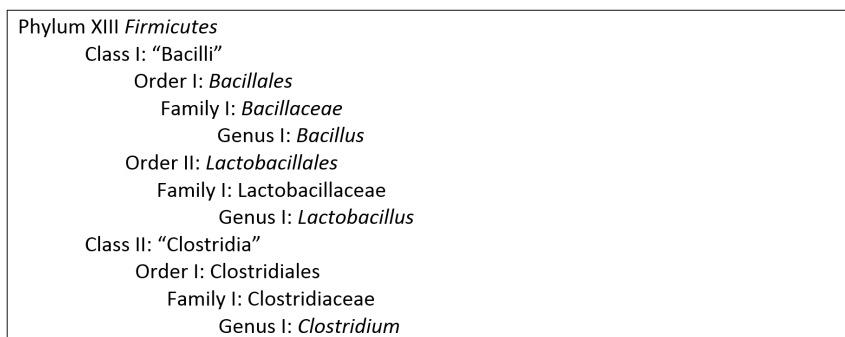


Figure 4 | Taxonomy of selected genera of the Phylum *Firmicutes* (adapted from Whitman, 2009).

a niche that has not been investigated widely to date. All of the samples studied were highly rich in taxa, which included not only the numerically dominant groups, but also rarer taxa, indicative of a very complex bacterial ecosystem. This data also revealed niche-partitioning among the treatments explored, from bagasse samples, floor dump sediments, through to cooling tower water. As discussed below, we propose that the habitat as a whole is remarkable in that it is highly selective for *Firmicutes*-type bacteria, and the shifts in the abundance of groups of *Firmicutes* (Classes, Orders etc) of these bacteria play a dominant role in defining the different habitat types.

This metagenomic study was designed to interrogate intact microbial communities, such as those found in the sugar mill samples, each of which comprises a separate micro-ecosystem. The intention of this research was to initiate a synecological approach to these communities, where a whole-of-community study would be used to determine such matters as Carbon degradation, Biofilm production, potential industrial processes to create useful end-products such as antibiotics or nutraceuticals, and other matters of interest. However, it is considered useful at this point to note some of the functional characteristics of the dominant Families in the majority of samples, in order to form a foundation for such community studies.

The presence of *Firmicutes* as a dominant group is quite rare for natural samples. The dominant Classes in the *Firmicutes* Phylum,

according to the data shown in Figure 3, were the *Bacilli*, *Catabacter*, *Clostridia*, the “gut clone group” and the *Mollicutes*. In all of the samples (treatments), by far the most abundant were the Class *Bacilli* and the Class *Clostridia*. The taxonomical position of these classes is shown in Figure 4. Some functional physiological information about each Class, and about examples of selected Genus/species from each Class, follows.

Members of the class Bacilli generally form a Gram-positive cell wall, may or may not form endospores, and grow aerobically, micro-aerophilically or as facultative anaerobes. The order *Bacillales* is delineated on 16S sequence analysis, and includes the families *Bacillaceae*, *Listeriaceae*, *Paenibacillaceae*, *Pasteuriaceae*, *Planococcaceae*, *Staphylococcaceae*, *Thermoactinomycetaceae*, and others. Many of this order have in common the characteristic of producing endospores, and are Gram-positive, at least in young cultures. The type genus for the order is *Bacillus*¹⁷. The genus *Bacillus* is characterized by cells which are Gram-positive, mostly straight rod-shaped, occurring singly, in pairs, and in chains¹⁷. They are generally aerobic endospore formers, the heat resistant endospore being arguably their most important characteristic. Endospores also confer resistance to radiation, disinfectant action, UV and desiccation, making the genus one of nature’s great survivors. The heat-resistant nature of the endospore accounts for the presence of *Bacillus* spp. in cooling tower

Table 4 | SIMPER analysis identifying which bacterial Classes contribute most strongly towards differences in FDS and CTW community compositions. (UC) denotes an unclassified class within the Phyla identified. Average abundance data is log-transformed, average PhyloChip probe intensity data (standardised over samples). Average dissimilarity is the contribution of each taxa towards dissimilarity between the two treatment groups (FDS and CTW). Dissimilarity is the average contribution/standard deviation of the contribution over groups (i.e. consistency)

Bacterial Class ¹	Average abundance ²		Average Dissimilarity ³	Dissimilarity/standard deviation ⁴	Contribution (%)	Cumulative contribution (%)
	FDS	CTW				
NC10-1 (UC)	0.59	0.66	0.04	1.87	2.84	2.84
Ellin6075/11-25	0.69	0.71	0.03	2.00	2.07	4.91
Acetobacterales	0.87	0.86	0.03	1.50	2.01	6.92
MND1 clone group	0.75	0.79	0.03	1.59	2.01	8.93
Oscillatoriales	0.59	0.58	0.03	1.14	1.97	10.90
Methylophilales	0.65	0.68	0.02	2.18	1.75	12.66
Hydrogenophilales	0.7	0.72	0.02	1.37	1.74	14.40
Bacillales	1.01	0.99	0.02	1.48	1.65	16.04
Lactobacillales	0.99	0.97	0.02	1.50	1.6	17.64
TM7 (UC)	0.67	0.68	0.02	1.66	1.53	19.17
Xanthomonadales	0.83	0.85	0.02	1.17	1.5	20.67
Burkholderiales	0.95	0.98	0.02	1.73	1.47	22.14
gut clone group	0.8	0.78	0.02	1.92	1.38	23.52
Proteobacteria (UC)	0.77	0.78	0.02	1.73	1.28	24.80
Chloroflexi-4 (UC)	0.71	0.68	0.02	1.30	1.22	26.03
Nitrosomonadales	0.79	0.81	0.02	1.71	1.19	27.22
Clostridiales	1.03	1.02	0.02	2.14	1.18	28.40
Plectonema	0.72	0.69	0.01	1.07	1.14	29.53



waters, as the heat exchange process selects for heat resistant micro-organisms, which become even more dominant as the water is recycled.

Using information from related research by this group, as yet unpublished, it was found by DGGE analysis and 16S rDNA sequencing (data not included), that the one of the major genera from the Phylum *Firmicutes* that were present in the liquid samples collected from sugar mills in Queensland, Australia, was *Bacillus subtilis* (found in all samples except one - CTW Maryborough). *B. subtilis* is known to use many sugars, its carbohydrate uptake and metabolism having been extensively reviewed by Deutscher *et al.* (2002)¹⁸. In fact, the majority of information regarding the metabolism and biochemistry of all *Bacillus* species relates specifically to *Bacillus subtilis*. An additional feature of *B. subtilis* is its ability to form biofilms, as a result of extracellular polysaccharide matrix production by the cells¹⁹. This capability is of great advantage to micro-communities, as the biofilm provides a matrix on which the community can develop.

The natural habitat of *B. subtilis* is soil, which contains a wide range of carbohydrates and polysaccharides from micro-organisms, plants and animals, and so it can utilize a wide range of such substrates and possesses a large number of enzymes which degrade polysaccharides. Interestingly, however, *B. subtilis* is also able to be isolated with ease from dried grass (“hay bacillus”). This may help to explain its major presence in the sugar mill samples, with sugar cane being a form of grass. The crushing and processing of sugar cane would release associated micro-flora, such as *B. subtilis*, into the mill environment, and the processing machinery would aid in the dissemination of aerosolized micro-flora throughout the mill, including into water sources such as the floor dump sumps and cooling towers. The common food-spoilage of sugar by “flat sours” refers to *Bacillus* species (Aerobic endospore formers), so its association with sugar processing is well recognized. The metabolic capabilities of *B. subtilis* make it an ideal organism to inhabit the sugar mill environment, as it is capable of metabolising the rich and diverse variety of sugars present.

The order *Lactobacillales* is distinguished by considerable gene loss, indicating early adaptation to nutritionally rich environments²⁰. Members of the order do, however, retain the genes for production of various specific antimicrobials (bacteriocins), demonstrating the long-term survival of *Lactobacillales* in complex microbial communities, such as those reported here. The members of this order are known for their sugar metabolism and energy conversion systems, using various parts of the glycolysis pathway, the pentose-phosphate pathway and the mannose-specific phosphotransferase pathway. Further metabolic abilities seem to be specific to particular members of the order. *Lactobacillales* are able to change from sole production of lactic acid to the production of mixed end-products^{21,22}. *Lactobacilli* commonly share the habitat when the content of sugar level is high in that habitat²³. It has been mentioned earlier that bagasse leachate contains very high levels of hemi-cellulose and residual hexose sugars. According to Kleerebezem and Vaughan (2009)²³, lactic acid bacteria are beneficial in the intestinal tract, producing lactic acid as an end-product of carbohydrate fermentation as well as bacteriocins²⁰. This ability has been exploited in food production owing to the growth-inhibiting effect that both acidification and bacteriocins have on spoilage agents. Since the lactic acid bacteria are generally regarded as safe (GRAS) for specifically defined uses and they often produce narrow spectrum antibacterial peptides active against pathogenic bacteria, they have also been used for health-promoting purposes as probiotics^{24,25}. Thus the presence of Lactic acid bacteria in sugar mill waste samples may have significant implications in the nutraceuticals and food industries. Like *Bacillus* species, many species of *Lactobacillus* are capable of producing biofilms. This capacity is advantageous in the probiotic industry, but is

also of importance to the environmental survival of *Lactobacillus* species in microbial communities²⁶.

The third member of the order *Firmicutes* to be examined is the genus *Clostridium*, type species *Clostridium butyricum*. Like other *Firmicutes*, *Clostridium* is characterised as being an endospore forming Gram-positive rod-shaped bacterium, but is distinguished by its anaerobic metabolism. *C. butyricum* is saccharolytic, producing butyric acid from glucose, as well as acetic acid, carbon dioxide and hydrogen gas. This species of *Clostridium* is “the dominating anaerobic spore-forming bacterium in certain silages”²⁷ and ferments a wide variety of carbohydrates, including polysaccharides such as starch and pectin. *C. butyricum* is one of a number of Clostridia under investigation for their ability to produce industrial solvents²⁷.

Summarizing the functional characteristics of the three dominant members of the Order *Firmicutes*, it can be seen that all three have wide-ranging metabolic capabilities, able to use various sugars as sole carbon sources. This ability is important in the niche environment being studied here. In addition, all three genera are able to produce useful end-products, particularly the *Clostridium* and *Lactobacillus* species. An interesting feature, given that these three genera are examples of the three dominant classes present in a single micro-environment, is the fact that one group is aerobic (*Bacillus*), one is microaerophilic (*Lactobacillus*) and the other is anaerobic (*Clostridium*). This is an ideal situation for a microbial community as the component genera are complimentary rather than competitive in their gaseous requirements. The ability of *B. subtilis* and the *Lactobacillus* sp. to form an extracellular matrix is also beneficial to the microbial community. Given the ability of *Clostridium* species to break down polysaccharides, it is interesting to note the potential dynamic – polysaccharide production and degradation occurring within the same community.

Proteobacteria were also one of the dominant groups present in all samples. The *Proteobacteria* Phylum includes a wide range of pathogens, such as *Escherichia coli*, *Salmonella* sp., *Vibrio* sp., *Helicobacter* sp. etc.²⁸. However, the majority of species in this Phylum are free-living, and also include a number of nitrogen fixing bacteria^{29,30}. The presence of the dominating soil Phylum such as *Proteobacteria*, *Bacteroidetes* and *Chloroflexi* in the environment is very common³¹.

Although the ecosystems were dominated by four major Phyla, a large overall diversity of taxa was present. The bagasse leachate sample was the most different in terms of community composition. The subsequent analysis revealed that the bacterial community was more diverse in bagasse leachate than FDS and CTW. This may be because of the temperature and composition of nutrients in the habitat. This information revealed that bagasse leachate is naturally enriched in carbon and nitrogen sources such as hemicelluloses¹. Lignocellulosic materials, such as sugar cane bagasse represent an abundant, inexpensive source of organic material which can be a carbon source for microbial growth¹. Sugar cane bagasse wastes are also sources of microbial nutrients (nitrogen and phosphate source). The micro flora is very likely to have the ability to grow in lignocellulose type substrates. The spent compost/cane bagasse mix, besides being an important source of nitrogen and phosphate nutrients, also provides an appropriate support matrix for native microorganisms.

In addition to *Firmicutes* being important discriminators of CTW versus FDS samples, a number of other Classes of bacteria were also found to be important. For example, Acetobacteriales, Oscillatoriales, Methylophilales, Hydrogenophilales, Bacillales, Lactobacillales, Xanthomonadales, Burkholderiales, Chloroflexi-4, Nitrosomonadales, Clostridiales and Plectonema. Some of these taxa have few cultured isolates; as such their wider ecology can only be inferred on the basis of environmental DNA sampling. Although these Classes were important in defining differences between the two habitat types, this information needs to be considered in light of the low



relative occurrence of many of the taxa. For the main part, the ecology of these ecosystems is dominated by the *Firmicutes*.

In order to confirm the data obtained in this study, the authors performed denaturing gradient gel electrophoresis (DGGE) followed by 16S rDNA sequencing on the same samples analysed by PhyloChip (unpublished data). The DGGE and sequencing results obtained confirmed the dominant genera in the *Firmicutes* phylum to be *B. subtilis*, *B. cereus* and *B. thuringiensis*. Other genera identified by DGGE and 16S rDNA sequencing representing the *Proteobacteria* phylum were *Brevibacillus brevis*, *Rhodospirillaceae* species, *Vibrio aestuarianus*, *V. vulnificus*, *Pseudomonas aeruginosa* and *P. fluorescens*. These are very important genera in various ways due to their physiological properties. For example genera capable of degrading hemicellulose include *Bacillus* species and *Pseudomonas* species, and genera capable of degrading cellulose and lignin such as *Pseudomonas* species. In addition, *Vibrio* species often degrade chitin and hemicelluloses. On the whole, *B. brevis*, *Rhodospirillaceae* species, *Bacillus* species, *Vibrio* species and *Pseudomonas* species are decomposers which have the ability to recycle nutrients in their niches. Protein producing *B. brevis* was found in all of the samples, and *Rhodospirillaceae* species are known to be photosynthetic bacteria which are responsible for oxygenation of the microbial environment.

Approaches based on metagenomic level analysis are needed if the complexity of these ecosystems was to be successfully explored. Amann *et al.*, (1995)³² and Craig *et al.*, (2010)³³ estimated that > 99% of microorganisms that were observable in nature typically were not able to be cultivated using standard culture techniques. The majority of the bacterial taxonomic divisions are poorly represented by cultured organisms³⁴. The overall performance of 16S rDNA sequence analysis is considered to be excellent, since it is able to resolve and almost identify the microbial species, when applied to a large collection of phenotypically unidentifiable bacterial isolates³⁵. Although 16S rDNA databases such as the arb-silva (<http://www.arb-silva.de/>), greengenes (<http://greengenes.lbl.gov/cgi-bin/nph-index.cgi>) and the ribosomal database project (RDP) database (<http://rdp.cme.msu.edu/>) have been developed to perform sequence comparisons and interpretation, further efforts should be made to expand 16S rDNA databases with high-quality sequences. This study has determined the general microbial community composition and potentially useful industrial micro-organisms in sugar mill cooling tower, bagasse leachate and floor dump sump liquid samples.

A high-density microarray system, PhyloChip used in this research to investigate the bacterial diversity and community composition of waste materials in three sugar cane processing plants in Australia. The samples investigated all harboured highly complex bacterial ecosystems. Four main Phyla dominated the ecosystems, with *Firmicutes* being the most dominant. This was followed by the Phyla *Proteobacteria*, *Bacteroidetes*, and then *Chloroflexi* according to the numbers. Within each sugar-cane mill, significant variation ($p < 0.05$) in community structure occurred between samples collected from the tested micro-niches, which were 'floor dump sump', 'cooling tower water', and 'bagasse leachate'. These differences were due to variation in a large number of bacterial Classes; however most were of low numerical abundance. Importantly, separation in community composition between these habitat types was also linked to Classes of *Firmicutes*, in particular the Bacillales, Lactobacillales and Clostridiales. The dominance of *Firmicutes* bacteria in these systems is likely to be linked to their physiology. As 'lactic acid bacteria', they are capable of fermentation of sugars present in these samples. This ability may assist in the displacement of other bacterial taxa, so providing a competitive advantage for *Firmicutes* bacteria.

Methods

Sites and sample collection. Sugar-cane mills were sampled from Proserpine (P), Mackay (MK) and Maryborough (M), all of which are located in Queensland, Australia. At each site, samples were collected from floor dump sump (FDS) and

cooling tower water (CTW). In addition, bagasse leachate (BGL) was collected from the Mackay mill, but this was not possible at the other two mills due to lack of rain. Samples of 250 mL were collected during spring (Oct – Dec), in sterile containers fitted with ice packs. All samples were kept at -20°C for short term use and long term storage was at -80°C .

DNA extraction and PhyloChip analysis of bacterial communities. DNA was extracted from all liquid samples including bagasse leachate using the ultra clean water DNA isolation kit (MOBIO laboratory Inc. USA) following the manufacturer's instructions. Extracted DNA was quantified by spectrophotometry (NanoDrop ND-100) and stored at -80°C until use.

A high-density oligonucleotide microarray system (PhyloChip G2) was used to taxonomically identify the bacterial community present in the liquid samples. The PhyloChip, based around the Affymetrix GeneChip platform (Affymetrix CA, USA), has probes covering 8,741 prokaryotic 16S rRNA OTU's PhyloChip^{6,7}.

Bacterial 16S rRNA genes were PCR amplified using primers 27F 5'(AGAGTTT GATCCTGGCTCAG)3' and 1492R 5'(GGTTACCTGTTCAGACTT)3'. PCR mixtures included primers at 0.3 μM each, dNTP's at 200 μM each, 1.2 U of Taq polymerase (Takara), 10 \times reaction buffers, 10 ng of template DNA and water to 25 μL . Eight individual PCR reactions were set up over a primer annealing range of 48–58 $^{\circ}\text{C}$ (reducing primer bias). After hot-start enzyme activation, PCR thermocycling consisted of 35 cycles of denaturation at 95 $^{\circ}\text{C}$ for 30 s, annealing for 30 s and with an extension at 72 $^{\circ}\text{C}$ for 90 s. A final elongation step was performed for 10 min at 72 $^{\circ}\text{C}$. PCR products from the separate 25 μL reactions were pooled, precipitated with isopropanol, washed with 80% ethanol and resuspended in water to concentrate the PCR products for PhyloChip analysis.

For each sample, 500 ng of PCR product was mixed with a control oligonucleotide spike and digested into 50–200 bp fragments with DNase I (Invitrogen) in One-Phor-All buffer (GE HealthCare). The 3' ends of the fragments were labelled with biotin using terminal deoxynucleotidyl transferase (Promega) according to the GeneChip DNA labelling procedure (Affymetrix, CA). The biotinylated mixture was denatured (99 $^{\circ}\text{C}$ for 5 min) and subsequently hybridised to a PhyloChip microarray at 48 $^{\circ}\text{C}$ and 60 rpm for 16 h. The hybridised array was washed and stained (streptavidin-phycoerythrin) on an Affymetrix fluidics station according to protocols described previously⁶.

Identification of taxa. Raw PhyloChip array data (CEL data output files from the Affymetrix GeneChip Operating System) was imported into PhyloTrac for processing⁸. Pixel images (fluorescence intensities in well defined grid) were resolved as probe pairs (perfect match (PM) and mismatch (MM)), which were grouped into probe sets (OTUs). Each probe set contains an average of 24 probe-pairs per OTU and also contains a central 17-mer not found in other OTUs. For each PM probe, a MM probe, with a single nucleotide difference was present. For each probe set, the trimmed mean fluorescence intensity (highest and lowest probe values removed before averaging) were normalized to internal spike-in control intensities using a maximum likelihood method, then scaled to the mean overall array and finally, log transformed. Taxa were considered present in a sample when at least 90% of the probes in its probe set pass the following criteria: PM/MM ≥ 1.3 and PM $\geq 130 \times$ background noise⁹.

A data file containing information on OTU's detected by the microarray, probe set intensity (mean value excluding the highest and lowest values), and hierarchical taxonomic classification was compiled. Manual reassignment of a few ambiguously-classified taxa was conducted (e.g. some *Bradyrhizobiales* were classified into the *Bradyrhizobiales* order and other *Bradyrhizobiales* into the *Rhizobiales*). A final tree, linking OTU intensity data in each sample to taxonomic rankings, was imported into the Primer 6 software package¹⁰ (PRIMER-E Ltd, UK) for analysis.

Microbial community analysis. Alpha-diversity was measured as both richness and evenness of the microbial communities, using selected indices as described by Kennedy & Smith (1995)¹¹. Richness of bacteria OTU's was measured across the taxonomic levels using Margalef's index (d). Similarly, community evenness was measured using the Pielou's index (J'); essentially the Shannon–Weiner index (H') divided by the maximum value H' can take.

$$d = \frac{(S-1)}{\log(N)}$$

$$J' = \frac{-\sum(p_i)(\log p_i)}{\log(S)}$$

Where S = number of 'species' (at a given taxonomic ranking), N = number of individuals, p_i is the proportion of intensity fluorescence for an individual OTU (at a given taxonomic level) relative to the sum of all the intensities detected in the sample.

When determining which taxa of bacteria are dominant in a system, the overall intensity data from the PhyloChip cannot be directly used past OTU (sub-family) level. Aggregation of this data to higher taxonomic levels imposes bias as probe coverage for bacteria such as *Proteobacteria* are much greater than those for 'rare' groups such as Phylum TM7. This can be overcome by using the 'top 100' taxa (probe intensity scores) for each treatment. The most abundant taxa are thereby selected and effects of differing coverage of probes across taxonomic groups are minimized. Accordingly, the top 100 probe intensity values were ranked for each treatment and



the combined data merged into a single file containing the top 100 for all treatments (total of 351 unique OTU). This data was used to explore which taxa were dominant in abundance across the treatments. Taxonomic aggregation of OTUs was performed in Primer 6¹⁰ as described previously.

Similarity in microbial community composition between samples was assessed to explore the effects the sample type and/or location effects. For each taxonomic level, OTU intensity values (full PhyloChip data set) were log-transformed and a resemblance matrix was generated using Gower's similarity method^{12,13}. The influence of 'sample type' and 'location' were then tested at each level of taxonomy using CAP analysis – canonical analysis of principal components – with probability testing using permutation (999 repeats). Ordination by non-metric, multidimensional scaling (nMDS) was used to visualise similarity in community composition (Class aggregated taxa) between samples¹⁴. As before, the underlying resemblance matrix for the nMDS was Gower's similarity measure. SIMPER analysis (similarity percentages/species contributions; data standardised) was used to determine which taxa were important in describing differences in taxa composition between the CTW and FDS treatments¹⁵. All multivariate data analysis in the Primer 6¹⁰/PERMANOVA software package (PRIMER-E Ltd., UK)¹⁶ using routines described previously^{10,14,16}.

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Author contributions

F.S. was responsible for sample collection, DNA extraction and co-writing the manuscript. S.W. performed the PhyloChip microarray experiment and assisted with data analysis. F.H., S.W. and M.H. also contributed to writing the manuscript and reviewed the manuscript.

Additional information

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