

Characterization of Microbial Diversity in Barley and Malt Using Terminal Restriction Fragment Length Polymorphism (T-RFLP)

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Introduction

Many intrinsic and extrinsic factors influence the composition and structure of the microbial community present in barley grains. Of these climate is believed to play an important role as a result of barley being cultivated in different geographic locations to produce have different microbial communities (Flannigan 2003). The aim of the present study is to determine the typical microbial composition and load of Australian malt and barley grown in different environments and areas, benchmarked against malting barley grown internationally using terminal restriction fragment length polymorphism (T-RFLP). T-RFLP is a rapid, sensitive, sequence-based technique for microbial

diversity assessment. The technique employed PCR and is used to amplify a selected region of genes encoding 16S rRNA for bacteria and 5.8S rRNA – ITS flanking regions for fungi (Monclavo *et al* 2000) from total community DNA. This method provides distinct profiles (fingerprints) dependent on the species composition of the communities of the samples and these fingerprints are often used to track the spatial and temporal changes in microbial diversity (Lüdemann *et al* 2000). This knowledge of microbial diversity would allow prediction and investigation of the likely beneficial and undesirable components in Australian barley and malt compared to their international counterparts.

Methodology

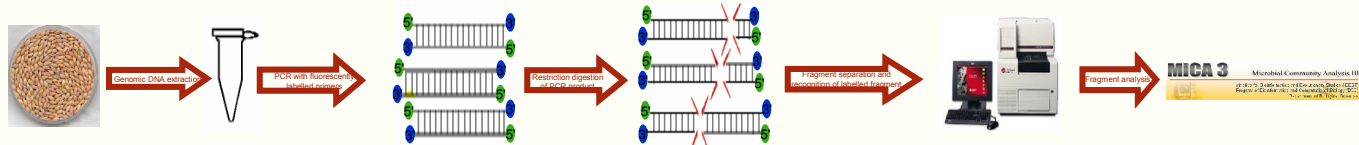


Figure 1. Outline of the T-RFLP method

In preliminary trials genomic DNA from ground barley and malt samples (0.1g) was extracted by modified FastDNA Spin Kit for Soil protocol (Laitila *et al* 2007). PCR was performed on extracted DNA samples with bacterial primers – 519f (5'-CAGCMGCCGCGTAATAC-3') and 1492r (5'-TACGGYACCTTGTACGACTT-3'). Both primers were labelled with Beckman WellRED D3 and D4 fluorochrome dyes (Sigma Genosys). PCR reactions were set up using HotStar Taq PCR kit (Qiagen, CA) and each 50µl reaction contained 2.5 units HotStar Taq DNA polymerase, 1µl PCR buffer (contained 15mM MgCl₂), 200µM of each dNTP, 0.5µl of each primer and 1µl of template DNA. Reactions were initially denatured for 15 min at 94°C followed by 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. This was followed by a final extension step of 72°C for 10 minute. The amplification products were assessed on a 1 percent agarose gel in 1xTAE buffer.

The PCR reactions were purified using UltraClean PCR clean up kit (Mobic, CA) according to the manufacturer's instructions, and purified DNA was eluted with 10mM Tris buffer. The purified amplicons were digested with RsaI, HhaI, MspI, HaeIII, and HinfI (New England Biolabs, Inc, MA) according to the manufacturer's instructions for 4, 16 and 21 hours at 37°C followed by enzyme inactivation at specified temperature for specified time followed by digestion clean up by ethanol precipitation. These restriction digestions were mixed with 0.25µl of WellRED fluorescent labelled GemoneLab600 internal size standard (GenomeLab/CEQ) and 10µl of formamide. Samples were loaded on CEQ 8000 series sequencer (Fig. 1) (Beckman Coulter, Inc, CA). A web based resource, microbial community analysis (MiCA) was used to study microbial community ecology (Shyu *et al* 2007).

Preliminary Results and Future Work

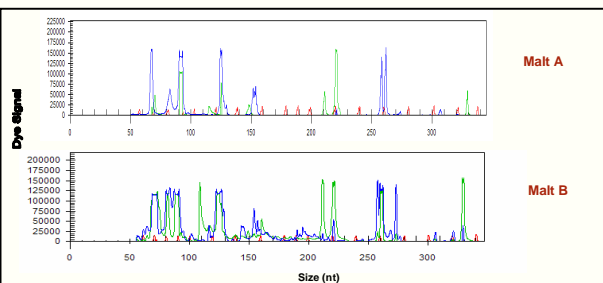


Figure 2. T-RFLP profiles from Hae III analysis of 16S rRNA gene PCR products amplified from DNA isolated directly from two malt samples (A & B).

The preliminary results demonstrated that T-RFLP is a robust and sensitive method for the rapid analysis of microbial community structure in different barley and malt samples. These results indicate that the study of community dynamics in response to different intrinsic and extrinsic factors is possible by this procedure. Distinct terminal fragments pattern in two malt samples showed unique fingerprints of bacterial microbiota associated with these samples (Fig. 2 & Table 1). As such these differing fingerprints could potentially indicate functional differences in malt quality.

Table 1. Bacterial diversity observed in two malt samples by analysing their T-RFLP profiles with MiCA database.

Malt A				Malt B			
Forward fragment	Reverse fragment	Abundance	Closest matching database OTU	Forward fragment	Reverse fragment	Abundance	Closest matching database OTU
70	261	0.179095	Eubacterium sp. C2	374	258	0.0084607	Uncultured bacterium SLB530
70	258	0.0634948	Desulfovibrio fructosovorans (T) DSM 3604	330	306	0.0058408	Uncultured bacterium SS-31
92	124	0.028549	Uncultured delta proteobacterium Dover132	211	126	0.0448676	Uncultured Vibrio sp. EC164
220	125	0.0268785	Escherichia coli CF T073	211	126	0.0448676	Araneobacera californiensis (T) Paocha-2
211	126	0.0287845	Uncultured Vibrio sp. EC164	139	220	0.0218959	Uncultured bacterium Raap110
211	126	0.0207845	Araneobacera californiensis (T) Paocha-2	117	126	0.0215154	Methylobacterium sp. RM892
92	126	0.022492	Uncultured bacterium DRV-SS8976	117	117	0.0213335	Flavobacterium sp. R21935
219	127	0.022282	Uncultured Kordirionids sp. BME62	127	126	0.0196863	Halomonadaceae bacterium MRN615
92	126	0.022247	Pseudomonas sp. GD100	127	126	0.0156883	Chromohalobacter salinarum CC 4.1
92	126	0.022247	Uncultured gamma proteobacterium 109 MERTZ2CM45	127	126	0.0156833	Enterobacter sakazaki E620
219	126	0.0220101	Klebsiella sp. XW111	329	125	0.0152029	Psychrobacter pacificensis NBH P2J13
219	126	0.0220101	Paritoea sp. BD 396.1	73	73	0.0148802	Frankia sp. S16-8
148	126	0.0210887	Uncultured Eryngomyxa sp. EC130	374	274	0.0122387	Uncultured candidate division OP3 bacterium 356 MERTZ2CM45
220	127	0.0208483	Uncultured bacterium QHC-828	110	72	0.0114818	Uncultured bacterium AP13U.307
329	125	0.0162746	Psychrobacter pacificensis NBH P2J13	110	72	0.0114818	Curvibacterium sp. VIM Ac-2057
83	125	0.0102417	Uncultured alpha proteobacterium 219 MERTZ2CM62	110	72	0.0114818	Frankia sp. M6J5
329	126	0.0097248	Lysobacter defluvi type strain: IMMB APB-9	93	273	0.0113471	Uncultured beta proteobacterium E089
329	126	0.0097248	Uncultured gamma proteobacterium Dover285	93	274	0.0113471	Paenibacillus chihensis JCM 8905
329	126	0.0097248	Enterococcus raffinosus LMG 12999	93	274	0.0113471	Paenibacillus sp. 2SS
218	125	0.00784567	Uncultured bacterium (human infant) S1G	406	273	0.0109708	Bornetia burgdorferi 272
218	125	0.00784567	Paritoea agglomerans A19	329	126	0.00984789	Lysobacter defluvi type strain: IMMB APB-9
67	124	0.00727764	Uncultured Verucomicrobia bacterium A.futuris15	329	126	0.00984789	Uncultured gamma proteobacterium Dover365
70	125	0.00657746	Methylobacterium sp. SKJH-20	329	126	0.00984789	Enterococcus raffinosus LMG 12999
70	124	0.00657746	Chromohalobacter funisensis Lil 2	138	73	0.00859007	Uncultured organism SP00204031
70	125	0.00657746	Acinetobacter sp. HJ2	110	121	0.00856419	Uncultured bacterium Pjy127
				373	72	0.00805264	Uncultured bacterium 425
				139	276	0.0083292	Uncultured bacterium 1.8
				93	73	0.00815968	Magnetically cocoon NP17 CS92
				93	72	0.00815968	Rothia sp. CCUG 26688
				93	72	0.00815968	Actinomyces sp. CCUG 26688
				93	72	0.00815968	Uncultured organism SP00204031
				73	128	0.00778718	Roseomonas gilardi subsp. gilardi (T) ATCC 49958
				73	128	0.00778718	Roseomonas gilardi subsp. gilardi ATCC 49958
				73	128	0.00741319	bacterium str. 47076
				73	128	0.00741319	Roseomonas gilardi subsp. gilardi EB464

Future work

- Standardize T-RFLP analysis for bacterial, fungal and yeast communities.
- Taxonomic identification and classification of bacteria, fungi and yeast using T-RFLP, clone libraries, plate culturing and/ or using group specific primers.
- Do comparative microbial analysis of Australian barley and malt with international counterparts for both their qualitative and quantitative compositions.
- Develop molecular markers for diagnosis of undesirable microbial contamination.
- Test the practical effect of these components on the brewing process and beer quality for impacts such as presence of mycotoxins, gushing, premature yeast flocculation (PYF) or unexpected changes in brewing performance.

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