

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

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Abstract

Our aim is to ensure that Australian malt and barley is 'clean' and free of undesirable microorganisms that may produce mycotoxins and factors that impact on malt quality, brewing process efficiency and beer quality. The study aimed 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 in which one of the two primers used is fluorescently labelled at the 5' end and is used to amplify a selected region of genes encoding 16S rRNA for bacteria and 5.8S rRNA – ITS flanking regions for fungi from total community DNA. 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, allowing further investigation focussed on the practical impact of these components on malt quality, and brewing process efficiency and beer quality. We expect that the study will highlight that Australian malt and barley is of high quality so that the health of domestic customers is ensured and it attracts a premium from our export customers.

Keywords: Malt, T-RFLP, Microbial diversity

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 (Ludemann *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

In preliminary trials genomic DNA from ground barley and malt samples (0.1g) was extracted by modified FastDNA Spin Kit for Soil protocol (Laitilal *et al* 2007). PCR was performed on extracted DNA samples with bacterial primers – 519f (5'-CAGCMGCCGCGGTAATAC-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-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 50ul reaction contained 2.5 units HotStar Taq DNA polymerase, 1mPCR buffer (contained 15mM MgCl₂), 200_μM of each dNTP, 0.5mL of each primer and 1mL of template DNA. Reactions were initially denatured for 15 min at 94oC followed by 35 cycles of 94oC for 1 min,

55oC for 1 min and 72oC for 1 min. This was followed by a final extension step of 72oC for 10 minute. The amplification products were assessed on a 1 percent agarose gel in 1mTAE buffer. The PCR reactions were purified using UltraClean PCR clean up kit (Mobio, 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 37oC followed by enzyme inactivation at specified temperature for specified time followed by digestion clean up by ethanol precipitation. These restriction digestions were mixed with 0.25_ L of WellRED fluorescent labelled GemoneLab600 internal size standard (GenomeLab/CEQ) and 10_ L of formamide. Samples were loaded on CEQ 8000 series sequencer (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

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. 1 & Table 1). As such these differing fingerprints could potentially indicate functional differences in malt quality.

Figure 1. T-RFLP profiles from Hae III analysis of 16S rRNA gene PCR products amplified from DNA isolated directly from two malt samples (A & B)

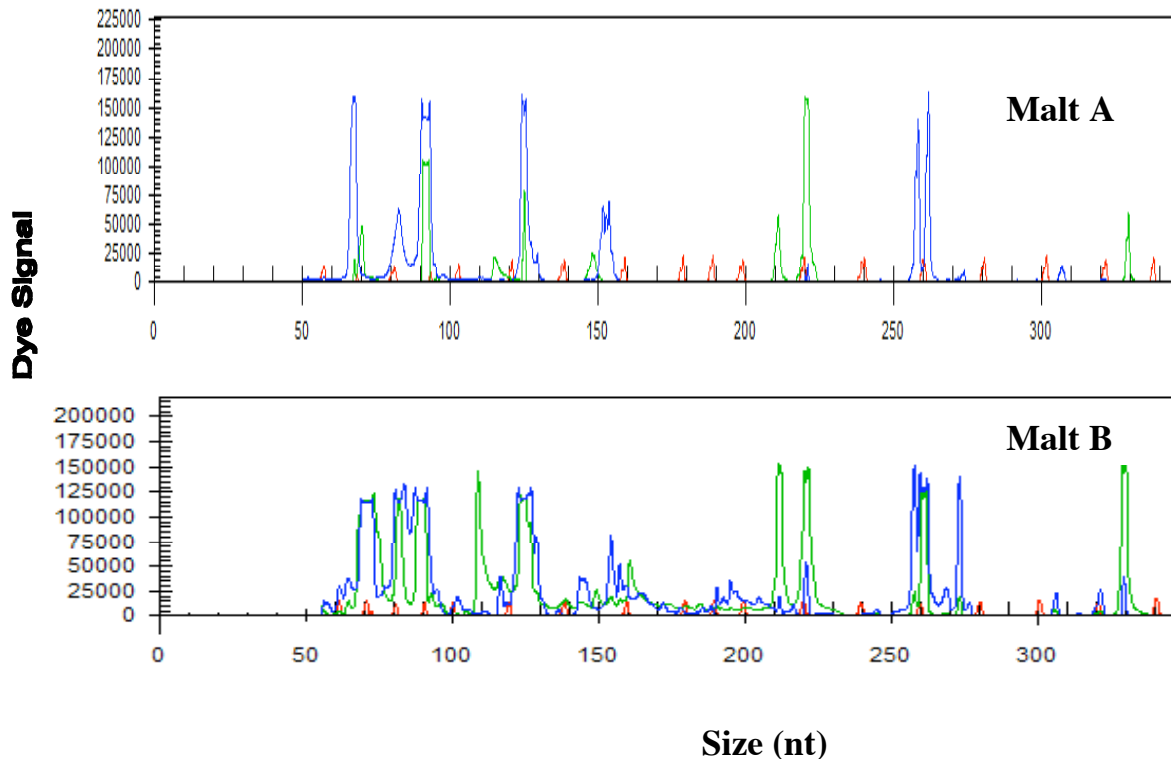


Table 1. Bacterial diversity observed in two malt samples (A & B) by analysing their T-RFLP profiles with MiCA database

Malt A	Forward fragment	Reverse fragment	Abundance	Closest matching database OTU
	70	261	0.179095	Eubacterium sp. C2.
	70	258	0.0634948	Desulfovibrio fructosovorans (T) DSM 3604.
	92	124	0.028549	uncultured delta proteobacterium Dover132.
	220	125	0.0268785	Escherichia coli CFT073.
	211	126	0.0267845	uncultured Vibrio sp. EC164.
	211	126	0.0267845	Anaerobranca californiensis (T) Paoha-2.
	92	128	0.022409	uncultured bacterium DRV-SSB076.
	219	127	0.022282	uncultured Kordiimonas sp. BME82.
	92	126	0.022247	Pseudomonas sp. GD100.
	92	126	0.022247	uncultured gamma proteobacterium 109 MERTZ21CM39.
	219	126	0.0220101	Klebsiella sp. XW111.
	219	126	0.0220101	Pantoea sp. BD 336 1.
	148	126	0.0210867	uncultured Enhygromyxa sp. EC130.
	220	127	0.0208483	uncultured bacterium QHO-B28.
	329	125	0.0162746	Psychrobacter pacificensis NIBH P2J13.
	93	125	0.0102417	uncultured alpha proteobacterium 219 MERTZ2CM62.
	329	126	0.00997248	Lysobacter defluvii type strain: IMMIB APB-9.
	329	126	0.00997248	uncultured gamma proteobacterium Dover385.
	329	126	0.00997248	Enterococcus raffinosus LMG 12999.
	218	125	0.00784567	uncultured bacterium (human infant) S1G.
	218	125	0.00784567	Pantoea agglomerans A19.
	67	124	0.00727764	uncultured Verrucomicrobia bacterium A.fistularis15.
	70	125	0.00657746	Methylobacterium sp. SKJH-20.
	70	124	0.00657746	Chromohalobacter tunisiensis Lit 2.
	70	125	0.00657746	Acinetobacter sp. HJ2.

Malt B	Forward fragment	Reverse fragment	Abundance	Closest matching database OTU
	374	258	0.0684667	uncultured bacterium SLB530.
	330	306	0.0508408	uncultured bacterium SS-31.
	211	126	0.0448676	uncultured Vibrio sp. EC164.
	211	126	0.0448676	Anaerobranca californiensis (T) Paoha-2.
	139	220	0.0218959	uncultured bacterium RsaP110.
	117	128	0.0215154	Methylobacterium sp. PMB02.
	117	117	0.0213335	Flavobacterium sp. R-21935.
	127	126	0.0156863	Halomonadaceae bacterium MRN515.
	127	126	0.0156863	Chromohalobacter salinarum CG 4.1.
	127	126	0.0156863	Enterobacter sakazakii E620.
	329	125	0.015209	Psychrobacter pacificensis NIBH P2J13.
	73	73	0.0148802	Frankia sp. Sn5-8.
	374	274	0.0122397	uncultured candidate division OP3 bacterium 356 MERTZ21CM45.
	110	72	0.0114818	uncultured bacterium API3U.307.
	110	72	0.0114818	Curtobacterium sp. VKM Ac-2057.
	110	72	0.0114818	Frankia sp. Mg15.
	93	273	0.0113471	uncultured beta proteobacterium EC69.
	93	274	0.0113471	Paenibacillus chibensis JCM 9905.
	93	274	0.0113471	Paenibacillus sp. 2S3.
	406	273	0.0109708	Borrelia burgdorferi 272.
	329	126	0.00984789	Lysobacter defluvii type strain: IMMIB APB-9.
	329	126	0.00984789	uncultured gamma proteobacterium Dover385.
	329	126	0.00984789	Enterococcus raffinosus LMG 12999.
	138	73	0.00959007	uncultured organism ctgBRRAA87.
	110	121	0.00956419	uncultured bacterium Flynn127.
	373	72	0.00905264	uncultured bacterium 425.
	139	276	0.0083292	uncultured bacterium I-8.
	93	73	0.00815998	magnetic coccus MP17 CS92.
	93	72	0.00815998	Rothia sp. CCUG 25688.
	93	72	0.00815998	Actinomyces sp. CCUG 25688.
	93	72	0.00815998	uncultured bacterium BF0002A031.
	73	126	0.00776718	Roseomonas gilardii subsp. gilardii (T) ATCC 49956.
	73	126	0.00776718	Roseomonas gilardii subsp. gilardii ATCC 49956.
	73	128	0.00741319	bacterium str. 47076.
	73	128	0.00741319	Roseomonas gilardii subsp. gilardii E9464.

Future Research

The differing fingerprints could potentially indicate functional differences in malt quality. The future plans are to standardize the T-RFLP analysis for bacterial, fungal and yeast communities. This will ensue taxonomic identification/classification of bacteria, fungi and yeast by using T-RFLP results in conjunction with clone libraries, plate culturing and/or using group specific primers. Comparative microbial analysis of Australian barley and malt with international counterparts for both their qualitative and quantitative microbial compositions will also be investigated. It is expected that the successful completion of this investigation will result in the development of molecular markers for diagnosis of undesirable microbial contamination. The project will also enable the testing of the practical effect of these microbial components on the brewing process and resultant beer quality for impacts such as presence of mycotoxins, gushing, premature yeast flocculation (PYF) or unexpected changes in brewing performance. Although these undesirable microbial impact have so far not been associated with Australian malt and malting barley, it is our aim to ensure that tests are developed to maintain this enviable reputation.

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