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ORIGINAL ARTICLE

Rapid identification of bacterial isolates from aqueous kava (*Piper methysticum*) extracts by polymerase chain reaction and DNA sequencing

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Abstract

Aim: Fresh kava beverages have a limited shelf life under refrigerated conditions. The objective of this study was to isolate and identify bacteria in aqueous extracts of kava rhizome.

Methods and Results: The internal part of kava rhizome was used to minimize soil contamination. Three kava extracts were prepared, serially diluted and plated on nutrient agar. Isolated colonies were identified by sequencing polymerase chain reaction amplicons targeting the eubacterial 16S rDNA and the *tuf* gene of *Staphylococcus*. Seventy-five bacterial isolates belonged to 16 genera. *Bacillus*, *Cellulomonas*, *Enterococcus*, *Pectobacterium* and *Staphylococcus* were identified in all kava extracts.

Conclusions: Kava rhizome contains large amounts of starch and fibre, which justify the presence of polysaccharide-degrading bacteria in the extracts. *Bacillus cereus* group and *Staphylococcus* species may produce toxins and cause food-borne illness.

Significance and Impact of the Study: The results of this study provide fundamental information that may be used to enhance the microbial quality and safety of kava beverages.

Introduction

Kava (*Piper methysticum*), a member of the pepper family Piperaceae, is a slow-growing perennial shrub. For over 3000 years, kava has been cultivated by Pacific islanders for its root system, the rhizome and roots. It usually takes 2–3 years for the root system to be mature enough for adequate accumulation of kavalactones. Kavalactones in small doses act as muscle relaxants and in large doses produce intoxication and sedation without loss of consciousness (Singh and Singh 2002; Whitton *et al.* 2003). Fresh and dried roots of the kava plant have been used to prepare nonalcoholic beverages, which are extensively used in social gatherings, rituals and ceremonies in the South Pacific region (Lebot *et al.* 1997).

However, the shelf life of fresh kava beverages is <2 days under refrigerated conditions, which makes it incompatible with today's food distribution system. Organoleptic changes in the spoiled beverage during refrigeration are the formation of starchy clumps and the emission of 'fruity' and 'putrid' odours. An effective pasteurization is difficult to achieve on kava beverages because of their high content of starch, which gels upon heating.

The microbial profile of kava beverages is still unknown. Organisms in the beverage are presumed to originate from kava rhizome or the processing environment. This study aimed to isolate and identify bacteria in aqueous extracts of kava rhizome. Kava rhizome is amorphous, modified stem tissue. It often contains cracks and holes. The inner portion of kava rhizome was used in the current study, because it is easier to clean than a whole rhizome or roots and is commonly used in kava bars to make kava beverages. Instead of traditional microbiological methods based on phenotypic and biochemical characterization, polymerase chain reaction (PCR) and DNA

sequencing were employed for rapid identification of the bacterial isolates.

Materials and methods

Sampling of kava rhizomes

Three kava rhizomes of 2 years old and 8–10 kg in weight were collected for this study. One sample was obtained from Oahu Island, and the other two were obtained from Hawaii Island, USA. The samples were transferred to the Food Microbiology Laboratory in the Department of Human Nutrition, Food and Animal Sciences, University of Hawaii at Manoa, for further analysis.

Preparation of kava extracts

All materials were autoclaved at 121°C for 15 min, except kava rhizomes. The rhizomes were separated from fibrous roots and thoroughly washed with running tap water until water was clear. The outer layer (*c.* 1 cm) of rhizomes was removed to minimize soil contamination. The inner portion was cut into small pieces of *c.* 2 cm in diameter. One thousand grams of kava pieces were mixed with 2000 ml of distilled water, blended in a kitchen blender at high-speed setting for 3 min and transferred to a nylon strainer bag. The edges of the bag were held together. Then, the bag was squeezed to collect the aqueous extract until no liquid could be squeezed from the residue. The pH of the kava extracts was measured immediately with a pH meter (Denver Instrument, Arvada, CO, USA).

Isolation of bacteria from kava extracts

The kava extracts were serially diluted using 0·1% peptone water. One hundred microlitres of appropriate dilutions was spread on nutrient agar (BD Diagnostic Systems, Sparks, MD, USA) and incubated at 37°C for 24 h. Bacteria were selected based on colony morphological characteristics such as colour, size, shape, margin and elevation, streaked on nutrient agar and incubated at 37°C for 24 h. After incubation, a single colony of each isolate was transferred to tryptic soy broth supplemented with 0·5% yeast extract (TSBYE; BD Diagnostic Systems) and incubated at 37°C for 24 h. This procedure was repeated twice to ensure the purity of bacterial isolates.

DNA extraction and PCR

DNeasy® Tissue kit (Qiagen, Valencia, CA, USA) was used to extract DNA from the cultures of purified isolates, according to the manufacturer's instructions. PCR was performed by using PCR Master Mix (Promega, Madison, WI, USA) in a Personal Mastercycler (Eppendorf, Hamburg, Germany). A reaction volume of 50 μl of PCR mixture contained 25 μl of 2× reaction mixture (400 μmol l^{-1} of each deoxynucleoside triphosphates, 3 mmol l^{-1} $MgCl_2$ and 1·25 U of *Taq* DNA polymerase), 0·5 μmol l^{-1} of forward and reverse primers and 5 μl of DNA extract from each bacterial isolate. 16S rDNA-based universal primers, modified from what Hall *et al.* described (2003), were employed in PCR for identification of the bacterial isolates from the kava extracts. For certain *Staphylococcus* isolates, primers targeting the *tuf* gene were used for further identification (Martineau *et al.* 2001). The name, melting temperature and sequence of the primers and corresponding PCR conditions are given in Table 1. Following the amplification, 5 μl of PCR products was electrophoresed through a 1% agarose gel in TAE buffer (0·04 mol l^{-1} Tris–acetate, 0·001 mol l^{-1} EDTA, pH 8·0). The gel was stained in SYBR® green (Invitrogen, Carlsbad, CA, USA) and visualized using FOTO/Analyst® Investigator System (Fotodyne, Hartland, WI, USA). PCR markers (Promega) were included in each gel to estimate the size of PCR products.

Sequencing and analysis of PCR products

The band of expected size in the agarose gel was excised under ultraviolet light. DNA was recovered from the

Table 1 Oligonucleotide primers used in the PCR

Primer	T_m (°C)	Sequence	PCR conditions	References
16S rDNA-F	57·3	5′-GGA GAG TTT GAT CCT GGC TCA G-3′	5 min at 95°C, 30 cycles of 30 s at 95°C, 30 s at 62°C and 45 s at 72°C, and 10 min at 72°C	(Hall *et al.* 2003)
16S rDNA-R	62·6	5′-TAT TAC CGC GGC TGC TGG CAC-3′		
tuf-F	61·9	5′-GGC CGT GTT GAA CGT GGT CAA ATC A-3′	4 min at 95°C, 30 cycles of 30 s at 95°C, 30 s at 55°C and 45 s at 72°C, and 7 min at 72°C	(Martineau *et al.* 2001)
tuf-R	54·0	5′-TI*A CCA TTT CAG TAC CTT CTG GTA A-3′		

*I, nucleotide analogue inosine.

gel slice using QIAEX® II gel extraction kit (Qiagen). Samples were sent to the Biotechnology Core Facility of University of Hawaii at Manoa, USA, and sequenced on an ABI PRISM™ 377 system (Perkin Elmer, Norwalk, CT, USA). To avoid amplification of artefact products, sequencing was performed from both the 5′ and the 3′ ends of each PCR product. Two sequences of sense and antisense strands were edited and assembled into a consensus sequence of corresponding amplicon. To determine the closest relatives of the partial 16S rDNA sequences or the partial *tuf* gene sequences, searches in the GenBank database were performed by using the BLAST program. Ninety-seven per cent similarity was used as the criterion for species identification (Stackebrandt and Goebel 1994). Scores of 96–97% similarity identified the belonging to the genus.

Results

Three aqueous extracts were made from the inner portion of fresh kava rhizomes. The source, aerobic plate count and pH of the kava extracts are given in Table 2. These extracts had a final pH ranging from 6·50 to 6·74. The near-neutral pH provides a favourable condition for microbial growth and poses a challenge in maintaining the quality of kava drink. The aerobic plate count ranged from 3·7 $\log_{10}$ CFU ml^{-1} in extracts 1–5·1 $\log_{10}$ CFU ml^{-1} in extract 2. Because the kava rhizomes were harvested from different locations in Hawaii, the bacterial level of kava extracts might reflect variations in soil microbial biomass. A total of 20, 28 and 27 isolated colonies were selected from kava extracts 1, 2 and 3, respectively, to represent all colony types (Table 3). The purity of the bacterial isolates was confirmed using the streak plate and Gram staining methods (data not shown).

DNA extracts from the purified bacterial isolates were subjected to a universal PCR capable of amplifying a highly variable region of the eubacterial 16S rDNA. Amplicons of 529 bp were generated from all the DNA extracts. Sequence analysis of the PCR products identified 65 out of 75 bacterial isolates from the kava extracts to the species level. Table 3 lists various bacterial species identified in this study and suggests considerable variation in the microbial profile of kava extract based on the source of raw materials. The bacterial isolates belonged to 16 genera including *Acinetobacter*, *Bacillus*, *Cellulomonas*, *Citrobacter*, *Enterobacter*, *Enterococcus*, *Erwinia*, *Klebsiella*, *Kocuria*, *Lactococcus*, *Microbacterium*, *Paenibacillus*, *Pantoea*, *Pectobacterium*, *Pseudomonas* and *Staphylococcus*. *Bacillus*, *Cellulomonas*, *Enterococcus*, *Pectobacterium* and *Staphylococcus* were identified in all kava extracts. Overall, the two extracts made from the kava rhizomes obtained from Hawaii Island were very similar in the microbial profile. *Kocuria*, *Microbacterium* and *Paenibacillus* were isolated only from extract 1, whereas *Citrobacter*, *Erwinia*, *Klebsiella*, *Lactococcus*, *Pantoea* and *Pseudomonas* were isolated only from extracts 2 and 3.

Three bacterial isolates from extract 1 could only be identified to the genus *Staphylococcus* by the 16S rDNA-based universal PCR. They were further analysed by sequencing PCR-amplified *tuf* gene fragments. The *tuf* gene, which encodes the elongation factor Tu (EF-Tu), is involved in peptide chain formation and is an essential constituent of the bacterial genome (Martineau *et al.* 2001). The *tuf* gene-based PCR yielded amplicons of 371 bp from the three *Staphylococcus* isolates. Sequence

Table 2 Source, bacterial count and pH of the kava extracts

Kava extract	Source	Aerobic plate count ($\log_{10}$ CFU ml^{-1})	pH
Extract 1	Oahu, HI	3·7 ± 0·07*	6·74 ± 0·04
Extract 2	Hawaii Island, HI	5·1 ± 0·04	6·50 ± 0·01
Extract 3	Hawaii Island, HI	5·0 ± 0·08	6·57 ± 0·02

*Standard deviation, results were averaged from two separate experiments.

Table 3 Bacterial isolates identified based on 16S rDNA sequence similarities to the closest relatives

Isolated organism	No. of isolates per extract 1	2	3	Closest relative	% Similarity
Acinetobacter	–	1	–	*Acinetobacter* sp.	97
Bacillus	3	3	2	*Bacillus cereus* group	99
	1	1	2	*Bacillus megaterium*	99
	1	–	–	*Bacillus fusiformis*	99
Cellulomonas	2	1	2	*Cellulomonas denverensis*	99
Citrobacter	–	1	2	*Citrobacter freundii*	99
Enterobacter	–	1	–	*Enterobacter aerogenes*	98
Enterococcus	–	1	1	*Enterococcus saccharolyticus*	100
	1	2	1	*Enterococcus casseliflavus*	100
Erwinia	–	1	3	*Erwinia* sp.	96
Klebsiella	–	2	1	*Klebsiella pneumoniae*	98
Kocuria	2	–	–	*Kocuria kristinae*	100
Lactococcus	–	2	2	*Lactococcus lactis*	98
Microbacterium	1	–	–	*Microbacterium* sp.	97
Paenibacillus	1	–	–	*Paenibacillus stellifer*	98
Pantoea	–	3	2	*Pantoea agglomerans*	98
Pectobacterium	2	1	2	*Pectobacterium carotovorum*	98
Pseudomonas	–	3	3	*Pseudomonas chlororaphis*	98
	–	1	–	*Pseudomonas oryzihabitans*	99
	–	–	1	*Pseudomonas* sp.	97
Staphylococcus	–	4	3	*Staphylococcus epidermidis*	99
	3	–	–	*Staphylococcus pasteuri*	98
	3	–	–	*Staphylococcus* spp.	100
Total isolates	20	28	27		

analysis allowed identification of the isolates as *Staphylococcus warneri*.

Discussion

There are different types of bacteria in the interior of plant roots. Chelius and Triplett (2000) reported the endophytic lifestyle of *Klebsiella pneumoniae* in maize root tissue. Four bacterial genera including *Bacillus*, *Flavobacterium*, *Micrococcus* and *Rathayibacter* dominated the endophytic community of canola (Germida *et al.* 1998). In this study, polysaccharide-degrading bacteria were found in all the extracts and may be associated with the inner portion of kava rhizome. Because dry kava rootstock consists of *c.* 43% starch and 20% fibre (Lebot *et al.* 1997), it is assumed that some of these polysaccharide-degrading bacteria may be endophytic. Species belonging to *Bacillus cereus* group have been associated with the foods rich in starch content. They produce amylase, which is responsible for exo-hydrolysis of 1,4-glucosidic linkages in starch (Priest 1977). *Bacillus megatherium* is known to produce several extracellular starch-hydrolysing enzymes such as beta-amylase, cyclodextrin glucanotransferase and dextranase. Cyclodextrin glucanotransferase catalyses the synthesis of cyclodextrins from starch, whereas dextranase is responsible for exo-hydrolysis of dextran to glucose (Priest 1977). *Pectobacterium carotovorum* can produce extracellular pectolytic enzymes that destroy the integrity of pectin (Andresen *et al.* 2007). *Cellulomonas denverensis* can ferment several complex and simple sugars and produce acids (Brown *et al.* 2005).

Lactic acid bacteria, such as *Enterococcus* and *Lactococcus*, were also identified in the kava extracts. These bacteria may ferment carbohydrates to lactic acid via homo-, hetero- or mixed fermentation pathways. *Enterococcus saccharolyticus* was isolated from extracts 2 and 3, whereas *Enterococcus casseliflavus* was isolated from all the kava extracts. Enterococci play an important role in the ripening of cheese through proteolysis, lipolysis and citrate breakdown, thus contributing to its typical taste and flavour (Coppola *et al.* 1990; Manolopoulou *et al.* 2003). *Lactococcus lactis* is commonly used as a starter culture in the manufacture of dairy products such as cheese, butter and buttermilk. Polysaccharide-degrading bacteria may utilize complex carbohydrates in kava beverages, resulting in the formation of simple sugars. In turn, lactic acid bacteria present in the beverage may further ferment simple sugars. The synergetic effect of these two types of bacteria may cause a drop in pH of kava beverages over time, which is a major factor that influences the quality of kava beverages. Moreover, *Pseudomonas* species were identified in the kava extracts made from the kava rhizomes acquired from Hawaii Island. *Pseudomonas* species are able to hydrolyse complex carbohydrates, and some of them are psychrotrophic. They are common causative agents of the spoilage of fresh foods stored aerobically at refrigerated temperature (Ercolini *et al.* 2007).

While most of the isolated bacteria are nonpathogenic, *B. cereus* group and *Staphylococcus* species may present a safety concern. Members of *B. cereus* group are soil inhabitants and ubiquitous in nature (Te Giffel *et al.* 1996). They may produce enterotoxins or an emetic toxin that can cause diarrhoea or vomiting (Pirhonen *et al.* 2005). Although both syndromes are relatively mild and do not last for more than 24 h, there were some extreme foodborne cases that led to death (Lund and Granum 1996; Mahler *et al.* 1997). Further, staphylococci are widely distributed in the environment and are associated with the skin, skin glands and mucous membranes of warm-blooded animals. Their presence in the kava extracts may reflect contamination of the kava rhizomes prior to the preparation of the beverages. The isolated *Staphylococcus* species included *Staphylococcus epidermidis*, *Staphylococcus pasteuri* and *Staph. warnerii*. They are coagulase negative. The majority of food poisoning has been attributed to the ingestion of enterotoxins produced by coagulase-positive *Staphylococcus aureus*. However, coagulase-negative staphylococci may also be enterotoxigenic (Vernozy-Rozand *et al.* 1996). Marín *et al.* (1992) reported that *Staph. epidermidis* isolated from dry-cured hams could produce enterotoxin C.

In conclusion, the occurrence of various bacteria in the aqueous kava extracts indicates considerable variation in the bacterial composition of kava rhizomes obtained from different locations in Hawaii. In addition to the polysaccharide-degrading bacteria, there are other organisms which may be contaminants. To our knowledge, this is the first report on the microbiological analysis of highly perishable kava beverages. It provides fundamental information that may be used to enhance the microbial quality and safety of kava beverages.

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