PE8.34 Detection of difficult cultivable bacteria from chicken carcasses using near-native medium 394.00

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Abstract Investigation of microbial spoilage in meats is usually hindered by the lack of suitable growth media and protocols to characterize the causative agents. A near-native medium of chicken extract agar (CEA) was developed by the addition of chicken extract into conventional plate count agar (PCA) to detect difficult cultivable bacteria on chicken carcasses, and the identification of recovered isolates growing only on CEA was performed by biochemical charactering coupled with 16S rDNA sequence analysis. Results showed that colony form unit (cfu) was significantly greater ($P < 0.05$) and the microflora was more diversified in CEA than those in PCA. CEA containing 30% (w/v) chicken extract gave the highest cfu of 64 CFU g-1, almost twice as that in PCA, and three bacteria absent on PCA were recovered. According to their morphology and biochemical feature, three bacteria were classified as Enterococcus sp, Rothia sp, and Staphylococcus sp respectively. The identification was confirmed by 16S rDNA sequence analysis with the similarity being 99% for Enterococcus faecalis and Staphylococcus saprophyticus subsp, and 96% for Rothia mucllaginosa. Three bacteria recovered by CEA are considered as either spoilage (Enterococcus), opportunistic pathogen (Rothia sp), or pathogen (Staphylococci) in foods, thus to cause problems with respect to food safety. The detection strategy utilized in this work should assist in the characterization of difficult cultivable spoilage microorganisms in other high protein food systems.

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I. INTRODUCTION

Pathogenic microorganisms and spoilage microorganisms are two major categories of microbial contamination[1][2]. The high standard of hygiene in meat processing generally results in meat products with low microbial levels at the time of manufacture[3]. Species identification can be assessed through the use of either culture-dependent or culture-independent methods. Nowadays community-level studies are relying more and more on culture-independent methods based on the direct analysis of DNA (or RNA) without any culturing step. However, several authors remarked [5][6][7][8] that culture-independent methods regularly fail to identify species obtained using culture-dependent methods due to species representing less than 1% of the total community were thought not to be visible in the non-culture profiles of the microbial community, thus the two types of methods may reveal different images of the same community. Therefore, these authors suggest that using a polyphasic approach, combining culture-dependent and culture-independent methods, may be worthwhile to obtain a more accurate view of the structure of the microbial community on food products. Differences in nutrient composition and concentration between the food system and the growth medium may lead to poor recovery of food borne microorganisms. For example, recovery of osmophilic yeasts from foods with high salt and sugar concentrations requires the use of non-conventional media[9]. Several authors have reported the finicky growth behavior of dressing microbiota on various microbiological media, the requirement for extended incubation (10 d), or the need for inclusion of additional ingredients, including fructose, to improve recovery[10][11]. And in a recent study by Waite et al, it was shown that ranch dressing based medium was helpful for the growth of fungi that was difficult to be cultivated in common PDA agar[12]. So the objectives of this study were (1) to develop a non-selective medium for contaminated bacteria in chicken carcasses to provide reproducible and strong growth, comparable to standard media; (2) to isolate difficult cultivable microorganisms associated with contamination events

with recovery on food-based growth media developed; and (3) to identify the contaminated microorganisms that only grow on the near-native media developed.

II. MATERIALS AND METHODS

A. Formulation of chicken extract agar (CEA) To prepare chicken extract, 50 g chicken meat were homogenized in 100 ml of sterile water after grinding using A11 basic Analytical mill (IKA Inc.), and immersed overnight at 4 °. After that, sterile supernatant was taken by filtering with gauze and 0.20 ¦Ìm Millex-FG filter (Millipore Inc.). Then 3, 4, 5, 6, 7 ml chicken extract were diluted by sterile water till 10 ml respectively. The isolation medium was a modified chicken extract agar (CEA) containing 10% chicken extract dilution in a half strength standard PCA. The basal medium was prepared with agar (SoBiGel, Bie and Berntsen, Roedovre, Denmark) and autoclaved before cooling to 55°C. Medium of 18 ml was poured immediately into sterile Petri dishes (90 mm) in which 2 ml sterile chicken extract was added already.

B. Microbiological analysis For culture-dependent analysis, chicken carcasses were obtained from local cold storage. Using aseptic techniques, 25 g of each sample (n = 3) were homogenized in 225 ml of sterile peptone saline (1 g of peptone and 9 g of NaCl per liter). After shaking at 230 rpm for 10 min with a stomacher, this suspension was serially diluted (1:10) in peptone saline. One milliliter dilutions were inoculated onto plate count agar (PCA) and chicken extract agar (CEA) in triplicate to obtain the total aerobic count. Plates were then incubated for 48 h at 35 °. Colonies developing on plates were counted.

C. Isolation and characterization of contaminated bacteria from chicken extract agar (CEA) Two new 90 mm Petri dishes were prepared, with one filled with PCA, and another CEA. The spaces of plates were divided and numbered correspondingly. All microorganisms recovered from CEA were inoculated into the two Petri dishes, one number corresponding to one bacterium. All plates were then incubated for 48 h at 30 °. Then microorganisms only recovered in Petri dish filled with chicken extract agar (CEA) were isolated. The isolates were further re-isolated to check their purity, and were examined microscopically for cellular morphology and bacterial isolates were analyzed for Gram reaction. Pure cultures were stored in 30% glycerol at -20 °.

D. BIOLOG analysis Prior to testing, all strains were sub-cultured on blood agar base No. 2 (CM 271, Unipath Ltd, Basingstoke, UK) supplemented with 7% sheep blood and incubated for 16 h at 30°C. A single colony of each organism was taken from the blood agar medium and spread on Biolog universal growth medium (BUGM) agar supplemented with 1% glucose. Plates were incubated for 16 h at 30°C. Single colonies were removed from the agar medium and placed in a test tube containing 18 ml of 0.9% sterile saline. An even suspension of inoculum was standardized using Biolog Turbidimeter and GP turbidity standards. The suspension (150 ¦Ìl) was dispensed into each well of the Biolog microplate, which were incubated at 30 ¡ãC and read by eye at 24 h. Databases with the Biolog software (Release version 3.50) were subsequently tested in a blind study.

E. 16S rDNA sequence analysis Genotypic characterization of isolated strains was performed with single purified colonies[13]. PCR reactions using primers Eu27F (5'-AGAGTTTGATCATGGCTCAG-3') and 1492R (5'-TACGGTTACCTTGTTACGACTT-3') (Lane, D., 1991) were performed in 25 ml volumes containing 2.5 ¦Ìl 10¡ÁPCR reaction buffer (Invitrogen), 1 ¦Ìl of each primer (Invitrogen), 2 ¦Ìl of dNTPs (Invitrogen), 0.5 ¦Ìl of Taq Polymerase (Invitrogen), 2 ¦Ìl MgCl2 (Invitrogen), 2 ¦Ìl of genomic DNA template and 14 ¦Ìl ddH2O. DNA cycling was carried out in a PTC-100 thermal cycler (MJ Research Inc.) with the following conditions: 94 °C for 3 min; 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1.5 min for 30 cycles; and a final extension at 72 °C for 10 min. The proper lengths of the inserts were evaluated on agarose gels (fragment comparison with a D2000 DNA marker (Takara Inc). And the nucleotide sequences were determined from the purified 1500 bp whole fragment of the 16S rDNA gene. The obtained 16S rDNA sequence was aligned to sequences in GenBank using the BLAST program. The aligned 16S rDNA sequences of the related species were retrieved from the NCBI nucleotide database. The program Clustal X (version 1.8) with default parameters was run for multiple sequence alignment. Phylogenetic and distance analysis of the aligned sequences was performed by the program MEGA (version 4.0). The resulting unrooted tree topologies were evaluated by bootstrap analysis of the neighbor-joining method based on 100 replications. F. Statistical analysis Significant differences between averages of duplicate measurements were evaluated by performing

one-way ANOVA at a confidence level of p = 0.05 with a posthoc Tukey test using SPSS 13.0 package.

III. RESULTS AND DISCUSSION

3. 1. Medium development and bacterial enumeration

The changes of bacterial enumeration were monitored by PCA medium and a series of CEA medium containing different concentration of chicken extract (Fig. 1). The mean colony form unit (cfu) on PCA were 38 cfu g-1, significantly lower ($P < 0.05$) than 46 cfu g-1 from CEA with 15% extract, while the latter only gave the least bacterial counts among CEA series medium. The cfu value on CEA medium increased with the climbing up of chicken extract concentration in general, with 30% giving the highest value of 64 cfu g-1 ($P < 0.05$). Further increase of chicken extract did not give the raise of colony form unit. In our preliminary study, chicken chunk was tried to be used directly as incubate media that was separated from meat sample above by a sterilized membrane to give the full nutrients for the growth of all microorganisms existing in chicken carcass samples, but the contamination of chicken carcass itself during the incubation was difficult to be controlled. Therefore chicken extract was added into PCA media to mimic the nutrient environment in chicken meat. Our results showed that bacteria recovery was improved due to minimizing microbial stress associated with the transfer from the chicken carcasses to conventional PCA media, with 30% (w/v) of chicken extract giving the best recovery rate and diversity. The exact mechanism how our modified medium improves the growth of spoilage bacteria is unclear; however, it is reasonably inferential that some difficult-cultivable spoilage bacteria can only utilize nutrients presenting in chicken carcass, thus they are able to grow on meats during storage but not on conventional medium by standard procedure.

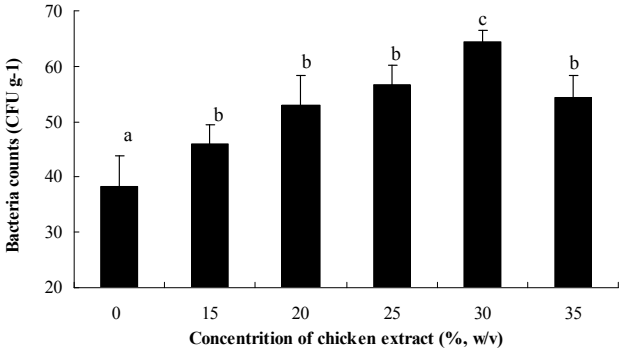

Fig. 1. Average microbial counts at medium containing different concentration of chicken extract dilution. a-c: differ ($P < 0.05$). Each point represents the mean value from three replicates. Vertical bars denote standard deviation.

3. 2. Isolation of chicken carcass microbes

A total of 133 colonies were isolated from CEA media. They were transferred to fresh PCA and CEA plates respectively to detect whether there were difficult cultivable bacteria that could only be recovered by our modified CEA medium. Finally three colonies only growing in CEA but not on PCA medium, named as CMA33, CMC18, CMC19, were obtained. Pure culture of three strains was acquired on CEA and the morphology of their colonies, including size, texture, and pigmentation was determined (Fig. 2 and Table 1).

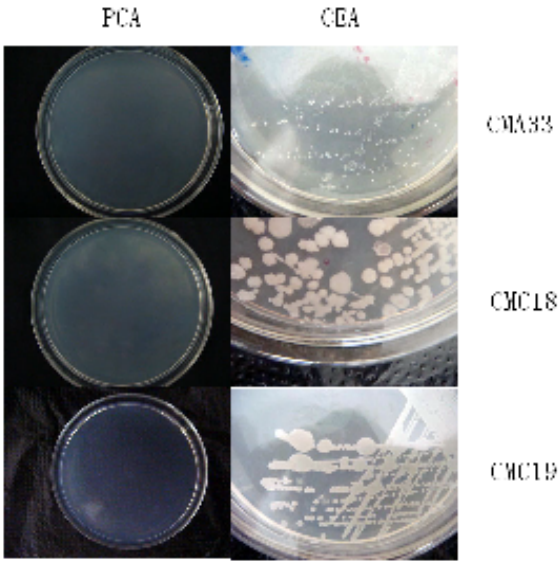

Fig. 2. Colony of three isolates growing only on CEA medium

It was clear that besides significantly increasing the colony form unit, the CEA medium developed in our study also could detect the bacteria that could not grow on conventional PCA medium. Accordingly, the modified CEA medium containing 30% of chicken

extract was chosen as the optimal near-native medium for the detection of bacteria on chicken carcass during storage.

Table 1 The properties of 3 special isolates

Characteristics of colony	Strains		
	CMA33	CMC18	CMC19
Gram staining	G^+	G^+	G^+
Shape	coccus	bacillus	coccus
Endospore	none	none	none
Form	circular	irregular	circular
Color	cream	pink	cream
Elevation	raised	raised	flat
Margin	entire	entire	entire
Texture	smooth	smooth	smooth
Appearance	dull	shiny	dull
Optical property	opaque	opaque	translucent

3. 3. BIOLOG system analysis Metabolic index was determined on Biolog microplate. It gave the utilization of 95 carbon substrates by three bacteria isolated on CEA (data not show). Biolog system analysis suggested that CMA33, CMC18, CMC19 be belong to Enterococcus, Rothia, and Staphylococcus respectively, with the probability index being 99%, 87%, 99% and similarity being 0.910, 0.576, 0.900. 3. 4. Identification of strains by 16S rDNA analysis The 16S rDNA fragment of 1.5 kb was amplified from the total DNA of each of three strains isolated on CEA medium and fully sequenced. After alignment with other 16S rDNA sequences in GenBank, it was disclosed that the CMA33 had a high degree of similarity (99%) to Enterococcus faecalis, the CMC18 strain to Rothia mucllaginosa (96%), and the CMC19 strain to Staphylococcus saprophyticus subsp (99%). The 16S rDNA sequence of the three bacteria was further aligned with the corresponding sequences. A phylogenetic tree based on all known representatives of validly described related species (data not shown). Genera Enterococcus, Rothia, Kocuria and Staphylococcus were included as reference strains of the three isolates, and the strains E. faecalis ATCC 19433, Rothia nasimurium sp. nov. CCUG 35957, S. saprophyticus subsp. ATCC 15305 included were those that exhibited the highest levels of similarity to CMA33, CMC18, CMC19. Enterococci are currently a controversial group of microorganisms due to its dual roles. Although they play an important role in the ripening of cheeses and the acquisition of their typical taste and flavor, the decarboxilating activity of some Enterococcus species can lead to the generation of biogenic amines (e.g. tyramine, histamine and tryptamine) from precursors, including tyrosine, hystidine and triptophane[14]. Rothia sp can use glucose to produce lactic acid and acetic acid. These species are also now recognized as opportunistic pathogen that can cause septicaemia and endocarditis, as well as other serious infections[15]. Staphylococcus are usually found in vacuum-packaged high protein foods. Lyhs[16] disclosed that S. epidermidis, S. hominis, S. sciuri, S. capitis, S. swarneri, S. intermedius and S. lentus were spoilage flora in vacuum-packaged rainbow trout. It was reported that anaerobic packaging of foods may increase the hazard of food poisoning since it allows toxin production by Staphylococcus while minimizes the activities of common spoilage microorganisms whose offensive changes can give warning of potentially unsafe conditions[17].

IV. CONCLUSION

Compared with the conventional culture methods and culture independent approaches, the improved near-native medium developed in current study may at times give better representation of the different bacterial species, especially those difficult cultivable spoilage microorganisms, present in meats. Further studies are needed to examine the eukaryotes existing in meat and meat products by this strategy.

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