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ORIGINAL ARTICLE

Comparison of the quantitative dry culture methods with both conventional media and most probable number method for the enumeration of coliforms and *Escherichia coli*/coliforms in food

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Significance and Impact of the Study: Current chromogenic media for coliforms and *Escherichia coli*/coliforms have enzymatic coloration due to breaking down of chromogenic substrates by food lactase. The novel sheet culture media which have film layer to avoid coloration by food lactase have been developed for enumeration of coliforms and *E. coli*/coliforms respectively. In this study, we demonstrated these media had comparable performance with reference methods and less interference by food lactase. These media have a possibility not only to be useful alternatives but also to contribute for accurate enumeration of these bacteria in a variety of foods, and specifically in fermented foods.

Keywords
chromogenic media, coliforms, coliforms/*E. coli*, dry culture media, enzyme, *Escherichia coli*, food safety, lactase.

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Abstract

Sanita-kun™ CC (coliform count) and EC (*Escherichia coli*/coliform count), sheet quantitative culture systems which can avoid chromogenic interference by lactase in food, were evaluated in comparison with conventional methods for these bacteria. Based on the results of inclusivity and exclusivity studies using 77 micro-organisms, sensitivity and specificity of both Sanita-kun™ met the criteria for ISO 16140. Both media were compared with deoxycholate agar, violet red bile agar, Merck Chromocult™ coliform agar (CCA), 3M Petrifilm™ CC and EC (PEC) and 3-tube MPN, as reference methods, in 100 naturally contaminated food samples. The correlation coefficients of both Sanita-kun™ for coliform detection were more than 0·95 for all comparisons. For *E. coli* detection, Sanita-kun™ EC was compared with CCA, PEC and MPN in 100 artificially contaminated food samples. The correlation coefficients for *E. coli* detection of Sanita-kun™ EC were more than 0·95 for all comparisons. There were no significant differences in all comparisons when conducting a one-way analysis of variance (ANOVA). Both Sanita-kun™ significantly inhibited colour interference by lactase when inhibition of enzymatic staining was assessed using 40 natural cheese samples spiked with coliform. Our results demonstrated Sanita-kun™ CC and EC are suitable alternatives for the enumeration of coliforms and *E. coli*/coliforms, respectively, in a variety of foods, and specifically in fermented foods.

Introduction

Coliform bacteria are important factors for assessing the sanitary conditions of food processing and, consequently, the food safety of the products because these bacteria are well-known indicators of faecal contamination (Gallagher and Spine 1968; Chung *et al.* 2000; Gray *et al.* 2002; Kang *et al.* 2003). Traditional methods for the estimation of numbers of coliform bacteria are the pour plate method, using conventional agar media and the 3-tube most

probable number (MPN) method as conventional methods (U.S. Food and Drug Administration, 2013). However, the pour plate method requires an experienced observer to discriminate typical coliform colony morphology. Furthermore, the MPN method is not suitable for daily hygiene control as it is too cumbersome and requires at least 5 days to obtain a result (AOAC International 2016). Over the past few decades, chromogenic media which contain enzyme substrates for β-galactosidase and β-glucuronidase have been used as suitable alternatives for the detection of coliform bacteria and *Escherichia coli* respectively (Kodaka *et al.* 1995; Geissler *et al.* 2000). This technology can easily discriminate and enumerate target organisms which possesses specific enzyme such as β-galactosidase since target organism forms specific coloured colony through the breaking down of specific substrate. However, these chromogenic media do not always work for fermented foods by enzymatic interference because these food samples make media discolour strongly through the breaking down of chromogenic substrates by lactase in these food samples. As a result, staining of media leads to unable to count accurately due to masking of target colonies.

On the other hand, to facilitate enumeration of coliform bacteria in food samples without preparing media, the 3M Petrifilm™ coliform count (CC) and *E. coli*/coliform count (EC) (PCC and PEC, respectively; 3M Company, Microbiology Products, St. Paul, MN) methods have been accepted as suitable ready-to-use alternatives as dry culture system (Ginn *et al.* 1986; Gangar *et al.* 1999). However, these media require a spreader device and confirmation of production of gas bubbles around colonies for count. Furthermore, production of gas bubbles on these media depends on strain of coliform bacteria.

To improve the ease of detection and enumeration without enzymatic interference, the Sanita-kun™ CC and EC methods (SkCC and SkEC, respectively; JNC Corporation, Tokyo, Japan), new ready-to-use dry sheet quantitative culture systems have been developed. Both SkCC and SkEC are based on the unique Sanita-kun™ system that consists of a transparent cover film, nonwoven fabric and water absorption polymer with growth medium components incorporated (Teramura *et al.* 2015). In addition, the multiple-layered medium structure avoids colour interference from lactase in the food itself in both SkCC and SkEC. This is achieved by separating the bottom layer with chromogenic substrates, nutrients and selective agents from the nonwoven fabric layer by a polyvinyl alcohol polymer film (Fig. 1A). As a result, coliform bacteria on the fabric layer grow selectively and absorb chromogenic substrates and nutrients from the bottom layer through the film following inoculation. As a result, there is less chromogenic interference by lactase present in the food samples (Fig. 1b,c) because lactase is prevented from contacting the chromogenic substrates directly. Furthermore, to ease operation, after inoculation of a 1-ml aliquot of sample onto both SkCC and SkEC, the sample is automatically diffused to the whole medium pad through the capillary action of the nonwoven fabric. Coliform bacteria grow and form blue-green/blue colonies from hydrolysis of by X-gal on SkCC after 24 h of incubation at 35°C. For SkEC, *E. coli* and other coliforms than *E. coli* grow and form purple/red-purple and blue-green/blue colonies from hydrolysis of Salmon-glucuronic acid and X-gal after 24 h of incubation at 35°C respectively. The ready-to-use chromogenic medium without enzymatic interference provides significant advantages for enumeration of coliforms from fermented foods. The aim of this study was to evaluate the performance of both SkCC and SkEC as new alternatives for enumeration of coliforms and *E. coli*/coliforms, respectively, relative to conventional culture methods.

Results and discussion

Inclusivity and exclusivity studies

Thirty coliform bacteria, 12 noncoliform Gram-negative bacteria, 33 Gram-positive bacteria and 2 yeasts were inoculated onto various media for inclusivity and exclusivity studies as shown in Table 1. A total of 30 coliform bacteria grew and produced blue-green/blue colonies on both SkCC and SkEC. The inclusivity of coliforms on SkCC was consistent with those on PCC, VRBA and Deso. On SkEC, PEC and CCA, a total of *E. coli* (4 strains of non-O157 strains) and one strain of *E. fergusonii*, both of which have β-glucuronidase, grew and formed the expected coloured colonies. Other coliforms tested than *E. coli* (non-O157 strains) and *E. fergusonii*, and two strains of *E. coli* O157 which lacks β-glucuronidase, grew and formed blue-green/blue, red and mauve colonies on SkEC, PEC and CCA respectively. For PCC and PEC, of 30 coliform bacteria tested, only 16 coliform bacteria had gas bubbles adjacent to the colony, even though all coliform bacteria grew and formed the expected coloured colonies. For noncoliform Gram-negative bacteria, no coloured colonies were observed on either SkCC or SkEC, whereas *Aeromonas hydrophila*, which has β-galactosidase (Ley *et al.* 1993), grew as coliform colonies on PCC, PEC, VRBA, Deso and CCA. For PCC and PEC, of 12 noncoliform strains, six strains (*A. hydrophila*, *Morganella morganii*, *Proteus mirabilis*, *P. vulgaris*, *Providencia alcalifaciens*, *Salmonella* Enteritidis and *S.* Typhimurium) grew and formed obvious red colonies similar to coliform colonies without gas bubbles. These results suggested that PCC and PEC cannot discriminate coliforms clearly in

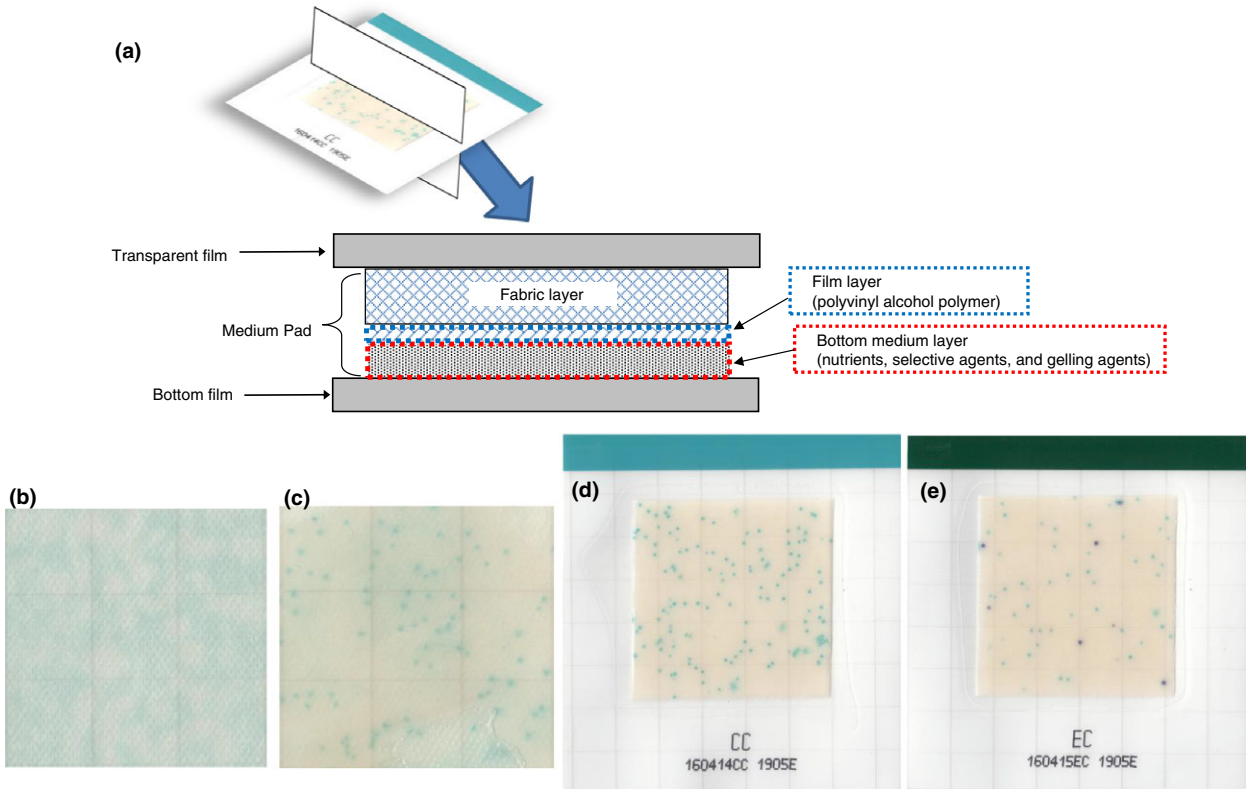

Figure 1 Basic structure of both the Sanita-kun™ coliform count and *Escherichia coli*/coliform count (SkCC and SkEC respectively) systems (a). The illustration depicts a vertical cross-sectional view. Appearance of enzymatic colour interference (b and c). Ten-fold dilutions of cheddar cheese samples spiked with *Enterobacter aerogenes* NBRC 13534 were inoculated onto the SkCC system without the film layer (b) and with the film layer (c). After 24 h of incubation at 35°C, SkCC without film (b) had interference from blue staining, whereas SkCC with film (c) could detect *E. aerogenes* as blue coloured colonies without colour interference. Typical appearance of colonies on both the SkCC and SkEC systems (d and e). *Enterobacter aerogenes* NBRC 13534 grew on SkCC (d) as blue-green/blue colonies after 24 h of incubation at 35°C. *Enterobacter aerogenes* NBRC 13534 and *E. coli* NBRC 102203 grew on SkEC (e) as blue-green/blue and purple/red-purple colonies, respectively, after 24 h of incubation at 35°C.

comparison with other methods, including SkCC and SkEC. Therefore, both PCC and PEC have a possibility of overestimation compared with other methods in coliform count. All Gram-positive bacteria (3 strains of *Bacillus* spp., 1 strain of *Corynebacterium*, 3 strains of *Enterococcus* spp., 2 strains of *Lactobacillus* spp., 2 strains of *Leuconostoc* spp., 2 strains of *Listeria* spp., 1 strain of *Micrococcus* sp., 18 strains of *Staphylococcus* spp. and 1 strain of *Streptococcus* sp.) and yeast strains (2 strains of *Candida* spp.) tested failed to grow under any of the reference methods conditions. Therefore, these results demonstrated that both SkCC and SkEC had excellent selectivity for coliform bacteria. According to ISO 16140 'Protocol for the validation of alternative methods', at least 30 positive and 20 negative strains should be used for inclusivity and exclusivity studies (ISO 2003). Our results indicate that 30 strains of coliform bacteria including *E. coli*, 12 strains of Gram-negative noncoliform bacteria, 33 strains of Gram-positive bacteria and 2 strains of yeast can be discriminated on both SkCC and SkEC. Therefore, the criteria of both inclusivity and exclusivity studies for enumeration of coliforms appear to be met.

Method comparison studies

The correlation coefficients (r), slopes, intercepts and mean log CFU per g with standard deviations (SD) of coliform counts recovered from 100 naturally contaminated food samples are presented in Table 2. The correlation coefficients (r) of SkCC with Deso, VRBA, CCA, PCC and MPN were 0·989, 0·991, 0·995, 0·997 and 0·954 respectively. Further, the correlation coefficients (r) of SkEC with Deso, VRBA, CCA, PEC and MPN were 0·989, 0·992, 0·994, 0·996 and 0·953 respectively. The slopes and intercepts determined by linear regression analysis between SkCC and Deso (slope, 1·00; intercept, 0·19),

Table 1 Colony appearances of Gram-negative bacteria tested on various methods*

Microbes tested†	SkCC	SkEC	PCC	PEC	VRBA	Deso	CCA
Coliform bacteria							
Cedecea lapagei JCM 1684	B	B	R	R	V	R	M
Citrobacter amalonaticus IFO 13547	B	B	R	R	V	R	M
C. koseri JCM 1659	B	B	R	R	V	R	M
C. freundii IFO 12681	B	B	R	R	V	R	M
Cronobacter sakazakii ATCC 12868	B	B	Rg	Rg	V	R	M
Cr. sakazakii ATCC 29544	B	B	Rg	Rg	V	R	M
Enterobacter aerogenes NBRC 13534	B	B	Rg	Rg	V	R	M
Ent. cloacae JCM 1232	B	B	R	R	V	R	M
Ent. cloacae IID 977	B	B	R	R	V	R	M
Ent. gergoviae JCM 1234	B	B	R	R	V	R	M
Escherichia coli NBRC 3972	B	RP	Rg	Bg	V	R	B
E. coli NBRC 13500	B	P	Rg	Bg	V	R	B
E. coli NBRC 15034	B	P	Rg	Bg	V	R	B
E. coli NBRC 102203	B	P	Rg	Bg	V	R	B
E. coli O157 ATCC 35150	B	B	Rg	Rg	V	R	M
E. coli O157 ATCC 43890	B	B	Rg	Rg	V	R	M
E. fergusonii NBRC 102419	B	P	Rg	Bg	V	R	B
E. hermannii JCM 1473	B	B	R	R	V	R	M
E. vulneris NBRC 102420	B	B	R	R	V	R	M
Hafnia alvei JCM 1666	B	B	Rg	Rg	V	R	M
Klebsiella oxytoca JCM 1665	B	B	Rg	Rg	V	R	M
K. pneumoniae JCM 1662	B	B	Rg	Rg	V	R	M
Kluyvera ascorbata JCM 1681	B	B	Rg	Rg	V	R	M
Klu. intermedia JCM 1238	B	B	R	R	V	R	M
Leclercia adecarboxylata NBRC 102595	B	B	Rg	Rg	V	R	M
Rahnella aquatilis IFO 13544	B	B	R	R	V	R	M
Raoultella planticola IFO 14939	B	B	R	R	V	R	M
R. terrigena NBRC 14941	B	B	Rg	Rg	V	R	M
Serratia marcescens JCM 1239	B	B	R	R	V	R	M
S. rubidaea NBRC 12973	B	B	R	R	V	R	M
Noncoliform bacteria							
Aeromonas hydrophila JCM 1027	C	C	R	R	V	R	M
Edwardsiella tarda JCM 1656	Not grown	Not grown	C	C	C	C	C
Morganella morganii IFO 3848	C	C	R	R	C	C	C
Proteus mirabilis JCM 1669	C	C	R	R	C	C	Br
Pro. vulgaris NBRC 3851	C	C	R	R	C	C	C
Providencia alcalifaciens IFO 12931	C	C	R	R	C	C	C
Pseudomonas aeruginosa IFO 3446	C	C	C	C	C	C	C
P. aeruginosa NBRC 12689	C	C	C	C	C	C	C
P. aeruginosa NBRC 13275	C	C	C	C	C	C	C
P. fluorescens NBRC 15842	Not grown	Not grown	C	C	C	C	C
Salmonella Enteritidis NBRC 3313	C	C	R	R	C	C	C
S. Typhimurium JCM 1652	C	C	R	R	C	C	C

*Characteristics indicate colony appearance: B, blue; RP, red-purple; P, purple; R, red; g, gas production; V, violet; M, mauve; C, colourless; Br, brown.

†Strains were derived from ATCC (American Type Culture Collection, Manassas, VA), IFO (Institute for Fermentation Osaka, Japan), IID (International Research Center for Infectious Diseases, Institute of Medical Science, The University of Tokyo), JCM (Japan Collection of Microorganisms) and NBRC (NITE Biological Resource Center, Japan).

SkCC and VRBA (slope, 1·03; intercept, −0·01), SkCC and CCA (slope, 1·03; intercept, −0·12), SkCC and PCC (slope, 1·00; intercept, 0·03) and SkCC and MPN (slope, 1·00; intercept, 0·42) were also close to 1·00 and 0·00 respectively. For SkEC, the slopes and intercepts determined by linear regression analysis with Deso (slope, 1·00; intercept, 0·19), VRBA (slope, 1·03; intercept, −0·01), CCA (slope, 1·03; intercept, −0·12), PEC (slope, 1·01; intercept, 0·01) and MPN (slope, 1·00; intercept, 0·42) were also close to 1·00 and 0·00 respectively. The

Table 2 Statistical relationship of SkCC and SkEC with reference methods for the enumeration of coliform in food samples

Parameter	SkCC *vs* Deso	SkCC *vs* VRBA	SkCC *vs* CCA	SkCC *vs* PCC	SkCC *vs* MPN	SkEC *vs* Deso	SkEC *vs* VRBA	SkEC *vs* CCA	SkEC *vs* PEC	SkEC *vs* MPN
No. of samples	100	100	100	100	100	100	100	100	100	100
Correlation coefficient (*r*)	0·989	0·991	0·995	0·997	0·954	0·989	0·992	0·994	0·996	0·953
Slope	1·00	1·03	1·03	1·00	1·00	1·00	1·03	1·03	1·01	1·00
Intercept	0·19	−0·01	−0·12	0·03	0·42	0·19	−0·01	−0·12	0·01	0·42
95% confidence limits	±0·298	±0·294	±0·295	±0·298	±0·296	±0·298	±0·294	±0·295	±0·298	±0·296
P value (ANOVA)*	0·38	0·64	0·95	0·86	0·05	0·38	0·64	0·95	0·83	0·05
Range (mean) of log CFU per g for 40 meat samples										
SkCC	1·00–5·87 (3·81)	1·00–5·87 (3·81)	1·00–5·87 (3·81)	1·00–5·87 (3·81)	1·00–5·87 (3·81)					
SkEC						1·00–5·75 (3·81)	1·00–5·75 (3·81)	1·00–5·75 (3·81)	1·00–5·75 (3·81)	1·00–5·75 (3·81)
Deso	1·18–5·78 (3·62)					1·18–5·78 (3·62)				
VRBA		1·00–5·57 (3·65)					1·00–5·57 (3·65)			
CCA			1·18–5·78 (3·84)					1·18–5·78 (3·84)		
PCC				1·00–5·85 (3·77)						
PEC									1·00–5·84 (3·77)	
MPN					0·56–5·04 (3·35)					0·56–5·04 (3·35)
Range (mean) of log CFU per g for 30 sea food samples										
SkCC	1·00–5·66 (3·00)	1·00–5·66 (3·00)	1·00–5·66 (3·00)	1·00–5·66 (3·00)	1·00–5·66 (3·00)					
SkEC						1·00–5·62 (2·99)	1·00–5·62 (2·99)	1·00–5·62 (2·99)	1·00–5·62 (2·99)	1·00–5·62 (2·99)
Deso	0·70–5·52 (2·77)					0·70–5·52 (2·77)				
VRBA		1·00–5·63 (2·94)					1·00–5·63 (2·94)			
CCA			1·00–5·65 (3·02)					1·00–5·65 (3·02)		
PCC				1·00–5·61 (2·95)						
PEC									0·70–5·57 (2·93)	
MPN					0·56–5·04 (2·53)					0·56–5·04 (2·53)
Range (mean) of log CFU per g for 30 vegetable samples										
SkCC	1·18–6·10 (4·54)	1·18–6·10 (4·54)	1·18–6·10 (4·54)	1·18–6·10 (4·54)	1·18–6·10 (4·54)					
SkEC						1·00–6·08 (4·54)	1·00–6·08 (4·54)	1·00–6·08 (4·54)	1·00–6·08 (4·54)	1·00–6·08 (4·54)
Deso	1·00–5·84 (4·39)					1·00–5·84 (4·39)				
VRBA		1·00–6·01 (4·48)					1·00–6·01 (4·48)			
CCA			1·18–6·03 (4·53)					1·18–6·03 (4·53)		
PCC				1·00–6·05 (4·51)						
PEC									1·18–6·04 (4·50)	
MPN					0·96–5·04 (4·22)					0·96–5·04 (4·22)
Mean log CFU per g ±SD (overall)										
SkCC ± SD	3·78 ± 1·53	3·78 ± 1·53	3·78 ± 1·53	3·78 ± 1·53	3·78 ± 1·53					
SkEC ± SD						3·78 ± 1·53	3·78 ± 1·53	3·78 ± 1·53	3·78 ± 1·53	3·78 ± 1·53
Deso ± SD	3·60 ± 1·51					3·60 ± 1·51				
VRBA ± SD		3·68 ± 1·47					3·68 ± 1·47			
CCA ± SD			3·80 ± 1·48					3·80 ± 1·48		
PCC ± SD				3·75 ± 1·52						
PEC ± SD									3·74 ± 1·51	
MPN ± SD					3·37 ± 1·46					3·37 ± 1·46

*$P > 0·05$ indicates no significant difference between both methods.

ranges of coliform numbers (log CFU per g) obtained from each tested methods in 40 meat, 30 seafood and 30 vegetable samples were 1·00–5·87, 1·00–5·66 and 1·18–6·10 (SkCC); 1·00–5·75, 1·00–5·62 and 1·00–6·08 (SkEC); 1·18–5·78, 0·70–5·52 and 1·00–5·84 (Deso); 1·00–5·57, 1·00–5·63 and 1·00–6·01 (VRBA); 1·18–5·78, 1·00–5·65 and 1·18–6·03 (CCA); 1·00–5·85, 1·00–5·61 and 1·00–6·05 (PCC); 1·00–5·84, 0·70–5·57 and 1·18–6·04 (PEC) and 0·56–5·04, 0·56–5·04 and 0·96–5·04 (MPN) respectively. The mean log CFU per g ±SD of SkCC, SkEC, Deso, VRBA, CCA, PCC, PEC and MPN for all samples were 3·78 ± 1·53, 3·78 ± 1·53, 3·60 ± 1·51, 3·68 ± 1·47, 3·80 ± 1·48, 3·75 ± 1·52, 3·74 ± 1·51 and 3·37 ± 1·46 respectively. The 95% confidence limits of the SkCC between Deso, VRBA, CCA, PCC and MPN were ±0·298, ±0·294, ±0·295, ±0·298 and ±0·296, respectively, whereas those of SkEC between each reference method were ±0·294 to ±0·298. Both SkCC and SkEC do not differ significantly ($P > 0·05$) from all reference methods in enumeration of coliforms by a one-way ANOVA as shown in Table 2.

For *E. coli* detection, method comparison study was conducted using artificially contaminated food samples because there was no sample which was sufficiently contaminated by *E. coli* in Japan. Table 3 shows correlation coefficients (r), slopes, intercepts and mean log CFU per g ±SD of *E. coli* counts recovered from 100 artificially contaminated food samples using *E. coli* detection media. The correlation coefficients (r) of SkEC with CCA, PEC and MPN were 0·978, 0·974 and 0·954 respectively. The slopes and intercepts determined by linear regression analysis between SkEC and CCA (slope, 0·95; intercept, 0·13), SkEC and PEC (slope, 0·96; intercept, 0·21) and SkEC and MPN (slope, 0·95; intercept, 0·19) were also close to 1·00 and 0·00 respectively. The ranges of *E. coli* numbers (log CFU per g) obtained from each tested methods in 40 meat, 30 seafood and 30 vegetable samples were 1·54–4·83, 1·30–3·88 and 1·40–3·92 (SkEC); 1·40–4·51, 1·00–3·96 and 1·54–3·87 (CCA); 0·70–4·59, 1·00–3·76 and 1·40–3·71 (PEC) and 1·36–4·38, 1·18–3·97 and 1·36–3·97 (MPN) respectively. The mean log CFU per g ±SD of SkEC, CCA, PEC and MPN for all samples were 2·65 ± 0·80, 2·65 ± 0·83, 2·55 ± 0·82 and 2·64 ± 0·82 respectively. The 95% confidence limits between SkEC and CCA, PEC and MPN were ±0·159, ±0·159 and ±0·159 respectively. SkEC did not differ significantly ($P > 0·05$) from all reference methods in enumeration of *E. coli* by a one-way ANOVA as shown in Table 3.

Table 3 Statistical relationship of SkEC with reference methods for the detection of *E. coli* in artificial contaminated food samples

Parameter	SkEC *vs* CCA	SkEC *vs* PEC	SkEC *vs* MPN
No. of samples	100	100	100
Correlation coefficient (*r*)	0·978	0·974	0·954
Slope	0·95	0·96	0·95
Intercept	0·13	0·21	0·19
95% confidence limits	±0·159	±0·159	±0·158
P value (ANOVA)*	0·96	0·39	0·91
Range (mean) of log CFU per g for 40 meat samples			
SkEC	1·54–4·83 (2·70)	1·54–4·83 (2·70)	1·54–4·83 (2·70)
CCA	1·40–4·51 (2·76)		
PEC		0·70–4·59 (2·63)	
MPN			1·36–4·38 (2·71)
Range (mean) of log CFU per g for 30 seafood samples			
SkEC	1·30–3·88 (2·63)	1·30–3·88 (2·63)	1·30–3·88 (2·63)
CCA	1·00–3·96 (2·54)		
PEC		1·00–3·76 (2·50)	
MPN			1·18–3·97 (2·58)
Range (mean) of log CFU per g for 30 vegetable samples			
SkEC	1·40–3·92 (2·61)	1·40–3·92 (2·61)	1·40–3·92 (2·61)
CCA	1·54–3·87 (2·60)		
PEC		1·40–3·71 (2·50)	
MPN			1·36–3·97 (2·60)
Mean log CFU per g ±SD (overall)			
SkEC ± SD	2·65 ± 0·80	2·65 ± 0·80	2·65 ± 0·80
CCA ± SD	2·65 ± 0·83		
PEC ± SD		2·55 ± 0·82	
MPN ± SD			2·64 ± 0·82

*$P > 0·05$ indicates no significant difference between both methods.

Therefore, these results suggest that both SkCC and SkEC are comparable in performance to all reference methods in enumeration of both coliforms and *E. coli*.

Assessment of inhibition of colour interference by food lactase

Both SkCC and SkEC, which have a specific culture medium structure, were compared with other similar chromogenic ready-to-use culture methods to assess the frequency of chromogenic interference by food enzymes using 40 artificially contaminated cheese samples. The mean CFU per plate, SD and number of samples stained are presented in Table 4. When 10-fold dilutions of spiked cheese samples were inoculated, the mean CFU per plate recovered from SkCC, SkEC, CDCF and CDEC were 49·6, 46·4, 32·7 and 26·9 respectively. Of 40 cheese samples, 6 (17·5%) samples caused enzymatic colour interference on the both SkCC and SkEC in enumeration of coliforms, whereas 13 (32·5%) samples caused interference on both CDCF and CDEC. There are significant differences ($P < 0·05$) between SkCC and CDCF, and SkEC and CDEC in counts by a one-way ANOVA as shown in Table 4, suggesting that counts on both SkCC and SkEC are higher than those on both CDCF and CDEC. Therefore, the culture medium structure of both SkCC and SkEC may have an effect not only on inhibition of enzymatic colour interference, but also on accurate enumeration of coliforms in fermented foods such as cheese. However, the frequency of interference may depend on the lactase concentration in the sample because interference was not completely blocked.

The layer of polyvinyl alcohol polymer film between the bottom medium layer and the nonwoven fabric layer acts as a filter to avoid contact of enzymes in the sample with chromogenic enzyme substrates in the medium layer. It has been suggested that coliform colonies could grow as coloured colonies because coliforms are able to transport enzyme substrates from the medium layer through the film layer actively and selectively. Both SkCC and SkEC were able to suppress enzymatic colour interference through this mechanism. This mechanism may be utilized as a chromogenic medium for other target organisms. Both SkCC and SkEC may provide significant advantages for food processing facilities as both SkCC and SkEC can decrease the frequency of colour interference and can enumerate coliform bacteria accurately from enzyme-containing food samples. Moreover, both SkCC and SkEC are simple to use and provide efficient culturing, as they are a presterilized and ready-to-use culture method. In this study, Sanita-kun™ CC (SkCC) and EC (SkEC), new dry sheet culture methods for enumeration of coliforms and *E. coli*/coliforms with a new medium structure, were evaluated. Both SkCC and SkEC had good correlation with and no significant differences ($P > 0·05$) from all reference methods for both coliforms and *E. coli* counts. Our results demonstrated that both SkCC and SkEC have excellent performance with less chromogenic colour interference. Therefore, both SkCC and SkEC would be useful alternatives for enumeration of both coliforms and *E. coli* in a variety of foods.

Materials and methods

SkCC and SkEC

After removing the transparent cover film, 1 ml of a sample was inoculated onto the centre of the nonwoven fabric pad. Coliform bacteria should form blue-green/blue colonies (Fig. 1d,e) on both SkCC and SkEC after 24 h of incubation at 35°C. *Escherichia coli* should form purple/red-purple colonies only on SkEC after 24 h of incubation at 35°C (Fig. 1e).

Inclusivity and exclusivity studies

Inclusivity and exclusivity studies were conducted using 77 microbes, including 30 coliform bacteria, 12 Gram-

Table 4 Recovery of coliform on various dry media in spiked cheese samples

Parameter	SkCC	SkEC	CDCC	CDEC
No. of samples	40	40	40	40
Mean CFU per plate	49·6	46·4	32·7	26·9
SD	20	18·5	11·1	13·3
No. (%) of staining samples	6 (17·5)	6 (17·5)	13 (32·5)	13 (32·5)
P value (ANOVA)*				
Between SkCC			1·23004E-05	
Between SkEC				7·02043E-07
Between CDCC	1·23004E-05			
Between CDEC		7·02043E-07		

*$P < 0·05$ indicates significant difference between both methods.

negative noncoliform bacteria, 33 Gram-positive bacteria and 2 yeast strains. After bacterial and yeast strains were cultured on tryptic soy agar (TSA; Difco, Becton Dickinson, Sparks, MD) at 35°C for 24 h, each culture was checked not to appear exogenous colony on TSA and then suspended in sterile phosphate-buffered saline (PBS) at the turbidity equivalent of a No.1 McFarland standard (*c.* 3·0 × 10^8 CFU per ml; McFarland 1907). Each inoculum strain was then serially diluted 10-fold in PBS. One millilitre of each suspension was cultured on SkCC, SkEC, deoxycholate agar (Deso; Difco), violet red bile agar (VRBA; Difco), Chromocult™ coliform agar (CCA; Merck KGaA, Darmstadt, Germany), PCC and PEC. The pour plate technique was used with Deso, VRBA and CCA following the protocol of the U.S. Food and Drug Administration (2013) *Bacteriological Analytical Manual.* Colony appearances on each medium were observed after 24 h of incubation at 35°C.

Food samples

For the method comparison study of enumeration of coliforms, 100 naturally contaminated food samples were purchased from retail stores in Yokohama city. The samples consisted of 40 meat samples, 30 seafood samples and 30 vegetable samples. For the method comparison study for detection of *E. coli*, the same foods were used as artificially *E. coli*-contaminated food samples. Three strains of *E. coli* (NBRC 3806, NBRC 15034 and NBRC 102203; NITE Biological Resource Center, Chiba, Japan) were used for spiking in the preparation of artificially contaminated foods. After each bacterial suspension was prepared as well as inclusivity and exclusivity studies, samples were randomly inoculated at low (1–2 log CFU per g), medium (2–3 log CFU per g) or high (3–4 log CFU per g) levels and were then stored for 3 days at 4°C. For assessment of the inhibition of enzymatic interference, 40 natural (nonprocessed) cheese samples, which were spiked with *Enterobacter aerogenes* NBRC 13534 at a low level (*c.* 2 log CFU per g), were stored for 3 days at 4°C.

Method comparison studies

For the method comparison study for enumeration of coliforms, 10 g of each sample was mixed with a ninefold volume of PBS in a sterile plastic bag (AZmax, Chiba, Japan) and homogenized for 90 s with a stomacher (MASTICATOR 400S, IUL, S. A., Barcelona, Spain). Each sample was serially diluted 10-fold with PBS. Dual measurements were then carried out for the following procedure. One millilitre of several dilutions of each food sample was inoculated onto SkCC, SkEC, Deso, VRBA, CCA, PCC and PEC. The pour plate technique was used for Deso, VRBA and CCA, following the protocol of the U.S. Food and Drug Administration *Bacteriological Analytical Manual.* Concurrently, the 3-tube MPN using lauryl tryptose broth (LST; Difco) and brilliant green lactose (BGLB) broth (Difco) was used as a reference following the protocol in *AOAC Official Methods of Analysis* 966·24 (AOAC International 2016). After 24 h of incubation at 35°C, colonies with the typical appearance of coliform colonies for each method tested were counted.

For the method comparison study for SkEC for detection of *E. coli*, sample preparation and inoculation were conducted as described above. The reference methods used were CCA, PEC and 3-tube MPN using LST and EC broth (Difco) with incubation at 45·5°C, following the procedures in *AOAC Official Methods of Analysis* 966·24. After 24 h of incubation at 35°C, colonies with the typical appearance of *E. coli* colonies (SkEC, purple/red-purple; CCA and PEC, blue) on each tested method were counted.

Assessment of inhibition of colour interference by food lactase

To assess colour interference by food lactase, 10 g of artificially contaminated cheese samples were mixed with a ninefold volume of PBS in a sterile plastic bag and homogenized for 90 s with a stomacher. One millilitre of each sample was inoculated directly onto SkCC and SkEC. The Compact Dry™ CF (CDCF; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and Compact Dry™ EC (CDEC; Nissui Pharmaceutical Co., Ltd.), which are chromogenic ready-to-use culture media, were used for comparison with SkCC and SkEC respectively (Kodaka *et al.* 2006a,b). After 24 h of incubation at 35°C, colonies typical in appearance for each method tested were counted as coliform, and the presence of coloration of medium by food lactase was visually observed for each tested method.

Statistical analysis

The results from the method comparison study were converted into log CFU per g of each tested food. All statistical analyses were performed with Microsoft Excel 2013 and evaluated at a significance level of 0·05. The linear correlation coefficients (r), slopes and intercepts between each tested methods were calculated. A one-way analysis of variance (ANOVA) was performed to determine differences in mean CFU counts of both SkCC and SkEC with each compared method.

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Conflict of Interest

The authors for this study declare that there is no conflict of interest.

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