pISSN 2733-4554
eISSN 2733-4562

J. Dairy Sci. Biotechnol. 2021;39(1):9-19
https://doi.org/10.22424/jdsb.2021.39.1.9

ARTICLE

Evaluation of Selective Media Containing Iron Source and Alpha-Glucosidase Substrates for *Enterobacter sakazakii* (*Cronobacter* spp.) Detection

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Received: March 9, 2021
Revised: March 19, 2021
Accepted: March 19, 2021

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Abstract

Enterobacter sakazakii (*Cronobacter* spp.) causes meningitis, necrotizing enterocolitis, sepsis, and bacteremia in neonates and children and has a high mortality rate. For rapid *E. sakazakii* detection, various differential and selective media containing α-glucosidase substrates, such as 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside (BCIG) or 4-methyl-umbelliferyl-α-D-glucoside (α-MUG), have been developed as only *E. sakazakii* exhibits α-glucosidase activity in the genus Enterobacter. However, *Escherichia vulneris* (family: Enterobacteriaceae) can also utilize α-glucosidase substrates, thereby resulting in false positives. Various iron sources are known to promote the growth of gram-negative bacteria. This study aimed to develop a selective medium containing α-glucosidase substrates for *E. sakazakii* detection that would eliminate false positives, such as those of *E. vulneris*, and to determine the role of iron source in the medium. Three previously developed (TPD) media, i.e., Oxoid, OK, and VRBG, and the medium developed in this study, i.e., NGTE, were evaluated using 58 *E. sakazakii* and 5 non-*E. sakazakii* strains. Fifty-four *E. sakazakii* strains appeared as fluorescent or chromogenic colonies on all four media that were assessed. Two strains showed colonies on NGTE medium and not on TPD media. In contrast, the remaining two strains showed colonies on TPD media and not on NGTE medium. None of the non-*E. sakazakii* strains showed fluorescent or chromogenic colonies on any of the evaluated media except *E. vulneris*, which showed colonies on TPD media and not on NGTE medium. This study demonstrated that the newly developed NGTE medium was not only equally efficient in promoting the growth of bacterial colonies when compared with the currently available media but also eliminated false positives, such as *E. vulneris*.

Keywords

Enterobacter sakazakii, selective media, glucose, α-glucosidase substrates, iron

Introduction

Previously referred to as 'yellow pigmented *E. cloacae*', *Enterobacter sakazakii* (*Cronobacter* spp.) was reclassified based on differences from *E. cloacae* in DNA relatedness, pigment production and biochemical reactions [1-5]. *E. sakazakii* (*Cronobacter* spp.) caused a severe form of neonatal meningitis with a high mortality rate [2]. A full risk assessment of *E. sakazakii* (*Cronobacter* spp.) will require more knowledge of its presence in food for neonates and infants [3]. Dried infant formula has been a vehicle of transmission in outbreaks and sporadic cases of *E. sakazakii* (*Cronobacter* spp.) [4]. Healy et al. [5] reported the enzymatic profiles of *E. sakazakii* (*Cronobacter* spp.) with specific reference to α-glucosidase reaction and concluded that *E. sakazakii*

(*Cronobacter* spp.) isolates produced α-glucosidases in contrast to other *Enterobacter* isolates. In 2002, US Food and Drug Administration published the Isolation and enumeration of *E. sakazakii* (*Cronobacter* spp.) from dehydrated powdered infant formula, but this method was very complicated and time-consuming procedures [6,7].

Therefore, to detect *E. sakazakii* (*Cronobacter* spp.) rapidly by using α-glucosidase reaction, Iversen et al. [8] made the new chromogenic (5-bromo-4-chloro-3-indolyl-α,D-glucopyranoside) medium, but the false positive showed 16 of 18 *Escherichia vulneris* strains, 2 of 3 strains of *Pantoea* spp., and 1 of 8 *Citrobacter koseri* strains on this agar [8]. Two major differences between *E. sakazakii* (*Cronobacter* spp.) and the other *Enterobacter* species are the presence of α-glucosidase and the absence of phosphoamidase in *E. sakazakii* (*Cronobacter* spp.) [8]. A fluorogenic method has been developed using α-glucosidase reaction, which was recognized a single, simple, and rapid test to distinguish *E. sakazakii* (*Cronobacter* spp.) from other *Enterobacter* species [8,9].

Leuschner & Bew [10] reported that a nutrient agar added with 4-methyl-umbelliferyl α-D-glucoside (α-MUG) as a differential-selective medium for the detection of *E. sakazakii* (*Cronobacter* spp.) was developed, but *E. vulneris* represented false positive on this agar. Guillaume-Gentil et al. [11] developed new media with addition of vancomycin to lauryl sulfate tryptose so as to detect *E. sakazakii* (*Cronobacter* spp.) in environmental samples, but some strains with yellow colonies were identified as *Pantoea* spp., *Pantoea agglomerans*, or *E. vulneris*.

Oh & Kang [12] reported α-MUG was used as a selective marker to develop a differential medium for *E. sakazakii* (*Cronobacter* spp.). And Restaino et al. [13] developed new choromogenic agar with contains two chromogenic substrates that are specific for *E. sakazakii* (*Cronobacter* spp.), plus selected sugars, a pH indicator, and inhibitors for both Gram-positive and Gram-negative contaminants all contributing to its selectiveityand differential properties. However, Oh & Kang [12] did not study the false positive as *Escherichia vulneris*, *Pantoea* spp., and *Citrobacter koseri* strains on this agar, and also Restaino et al. did not research the false positive as *Escherichia vulneris*.

Hence, it is necessary to develop the media for enrichment of *E. sakazakii* (*Cronobacter* spp.) or for recovery of injured *E. sakazakii* (*Cronobacter* spp.) without the false positive such as *E. vunleris*. It has been shown in numerous studies that supplementation of egg contents with iron such as ferric ammonium citrate (FAC), ferrous sulphate (FS) and ferrioxamine E (FE) can overcome the antimicrobial properties of ovotransferrin and enhance the growth and detection of *Salmonella* in eggs [14]. No strains of *E. coli*, the *Proteus-Providencia-Morganella*-group have an uptake and utilization system of FE [14,15]. However, Ferrioxamine E can be used by *Citrobacter spp., Klebsiella spp., Pseudomonas spp., Entrobacter spp.*, and *Yersinia enterocolitica* [15]. Hence, Ferrioxamine E is considered a selective iron source because it is utilized by *E. sakazakii* (*Cronobacter* spp.), but not by *E. coli*. Also, the type of carbohydrate was related with α-glucosidase reaction and production of yellow pigment.

This study was performed to distinguish *E. sakazakii* (*Cronobacter* spp.) from non-*E.*

sakazakii (*Cronobacter* spp.) on 4 different selective media for *E. sakazakii* (*Cronobacter* spp.), and to remove false positive such as *E. vulneris* and to compare the colony morphology of *E. sakazakii* (*Cronobacter* spp.) and non-*E. sakazakii* (*Cronobacter* spp.) on various modified selective media for *E. sakazakii* (*Cronobacter* spp.) made by combining iron sources and carbohydrate sources.

Materials and Methods

1. Strains

Strains of *E. sakazakii* (*Cronobacter* spp.) were obtained from a range of culture collections. A list of all strains with their origin is collected in Table 1. All strains were grown on tryptic soy broth (TSB: Becton Dickinson, USA) at 37℃. Purity of the culture was confirmed using a biotyping kit (API 20 E: bioMerieux) and real-time PCR.

Table 1. Chromogenic or fluorogenic reaction of 4 different media supplemented with α-glucosidase substrates in 58 strains of E. *sakazakii* (*Cronobacter* spp.)

Species	Strain no.	Source	Chromogenic or fluorogenic reaction			
			Oxoid	OK	VRBG	NGTE
E. sakazakii	FSM 145	UGA	+	+	+	+
E. sakazakii	FSM 261	NRC	+	+	+	+
E. sakazakii	FSM 262	NRC	+	+	+	+
E. sakazakii	FSM 265	NRC	+	+	+	+
E. sakazakii	FSM 270	NRC	+	+	+	+
E. sakazakii	FSM 271	NRC	+	+	+	+
E. sakazakii	FSM 272	NRC	+	+	+	+
E. sakazakii	FSM 273	NRC	+	+	+	+
E. sakazakii	FSM 274	NRC	+	+	+	+
E. sakazakii	FSM 275	NRC	+	+	+	+
E. sakazakii	FSM 287	NRC	+	+	+	+
E. sakazakii	FSM 290	NRC	+	+	+	+
E. sakazakii	FSM 292	NRC	+	+	+	+
E. sakazakii	FSM 293	NRC	+	+	+	+
E. sakazakii	FSM 294	NRC	+	+	+	+
E. sakazakii	FSM 295	NRC	+	+	+	+
E. sakazakii	FSM 297	NRC	+	+	+	+
E. sakazakii	FSM 298	NRC	+	+	+	+
E. sakazakii	FSM 299	NRC	+	+	+	+
E. sakazakii	FSM 300	NRC	+	+	+	+
E. sakazakii	FSM 302	NRC	+	+	+	+
E. sakazakii	FSM 303	NRC	+	+	+	+
E. sakazakii	FSM 318	NRC	+	+	+	+
E. sakazakii	FSM 321	NRC	−	−	−	+
E. sakazakii	FSM 324	NRC	+	+	+	+
E. sakazakii	1	UGA	+	+	+	+
E. sakazakii	1.91	UGA	+	+	+	+
E. sakazakii	2	UGA	+	+	+	+
E. sakazakii	2.39	UGA	+	+	+	+
E. sakazakii	2.40	UGA	+	+	+	+
E. sakazakii	2.41	UGA	+	+	+	+
E. sakazakii	2.42	UGA	+	+	+	+

Table 1. Continued

Species	Strain no.	Source	Chromogenic or fluorogenic reaction			
			Oxoid	OK	VRBG	NGTE
E. sakazakii	2.43	UGA	−	−	−	+
E. sakazakii	2.44	UGA	+	+	+	+
E. sakazakii	2.45	UGA	+	+	+	+
E. sakazakii	2.46	UGA	+	+	+	+
E. sakazakii	2.47	UGA	+	+	+	+
E. sakazakii	2.68	UGA	+	+	+	+
E. sakazakii	2.69	UGA	+	+	+	+
E. sakazakii	2.70	UGA	+	+	+	+
E. sakazakii	2.71	UGA	+	+	+	+
E. sakazakii	2.72	UGA	+	+	+	+
E. sakazakii	2.73	UGA	+	+	+	+
E. sakazakii	2.74	UGA	+	+	+	+
E. sakazakii	2.75	UGA	+	+	+	+
E. sakazakii	2.76	UGA	+	+	+	+
E. sakazakii	2.77	UGA	+	+	+	+
E. sakazakii	2.78	UGA	+	+	+	+
E. sakazakii	2.79	UGA	+	+	+	+
E. sakazakii	2.80	UGA	+	+	+	−
E. sakazakii	2.81	UGA	+	+	+	+
E. sakazakii	2.82	UGA	+	+	+	−
E. sakazakii	2.83	UGA	+	+	+	+
E. sakazakii	2.84	UGA	+	+	+	+
E. sakazakii	3	UGA	+	+	+	+
E. sakazakii	5	UGA	+	+	+	+
E. sakazakii	6	UGA	+	+	+	+
E. sakazakii	7	UGA	+	+	+	+

Oxoid, *BRILLIANCE* ENTEROBACTER SAKAZAKII AGAR (DFI formulation, Code: CM1055).
OK, Manufacture media using the method of Oh & Kang (2004) [12].
VRBG, violet red bile glucose.
NGTE, The newly developed medium in this study.
UGA, Dr. Jeffrey Kornacki, University of Georgia, Athens, GA; NRC, Dr. John Marugg, Nestle Research Center, Lausanne, Switwerland.
+, *E. sakazakii* (*Cronobacter* spp.); −, non-*E. sakazakii* (*Cronobacter* spp.).

2. Medium

Four different media were prepared to detect *E. sakazakii* (*Cronobacter* spp.) and non-*E. sakazakii* (*Cronobacter* spp.) by use of the enzyme substrate, 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside (BCIG) or 4-methyl-umbelliferyl α-MUG (Sigma-Aldrich, USA) and of various iron sources, FAC, FS, and FE (Table 2). When the agar reached a temperature of 50℃-60℃, enzyme substrate and iron sources were added to the agar.

3. Reaction of α-glucosidase substrates in 4 different media

The reaction of α-glucosidase substrates in 4 different media was determined by change of colony color under normal light or colony fluorescence under UV light after incubation at 37℃ for 24 h. Sensitivity was defined as the number of true positives divided by the sum of true positives plus false negatives, expressed as a percentage.

Table 2. Media used in this study

		Oxoid	OK	VRBG+α-MUG	NGTE+α-MUG
Reaction of *E. sakazakii* (*Cronobacter* spp.)	Normal light	Blue-green	Yellow	Pink	Yellow
	UV light	N/A	Fluorescent	Fluorescent	Fluorescent
Ingredients		5 g tryptone 5 g soya peptone 5 g sodium chloride 1 g ferric ammonium citrate 1 g sodium desoxycholate 1 g sodium thiosulphate 0.1 g chromogen 15 g agar	20 g tryptone 1.5 g bile salts no. 3 1.0 g sodium thiosulfate 1.0 g ferric citrate 50 mg α-MUG 15 g agar	7 g peptone 3 g yeast extract 1.5 g bile salts no. 3 10 g lactose 5.0 g sodium chloride 0.03 g neutral red 0.002 g crystal violet 50 mg α-MUG 15 g agar	5 g sodium chloride 10 g glucose 20 g tryptone 200 ng/mL fe 50 mg α-MUG 15 g agar

NGTE, The newly developed medium in this study.

Results

Fifty-eight strains of *E. sakazakii* (*Cronobacter* spp.) were analyzed using 4 different media supplemented with α-glucosidase substrates, BCIG or 4-methyl-umbelliferyl α-MUG. All strains formed colonies and the chromogenic or fluorogenic reaction are shown as Table 1. Fifty four *E. sakazakii* (*Cronobacter* spp.) strains appeared as fluorescent or chromogenic colonies on all of the media tested.

Two strains were negative on Oxoid, OK, and VRBG media while showing positive on newly developed medium (NGTE) medium. Interestingly, the other two strains were observed vice versa showing positive on Oxoid, OK, and VRBG media, but negative on NGTE medium (Table 3). The detection rate of *E. sakazakii* (*Cronobacter* spp.) on each medium expressed over 96% recovery (Table 4). The comparison of colony size on the media with addition of various iron sources showed in Table 5.

In 58 *E. sakazakii* (*Cronobacter* spp.) tested, all 4 different media showed over 96% recovery rate (Table 4). Each colony of each media inoculated *E. sakazakii* (*Cronobacter* spp.) was verified as *E. sakazakii* (*Cronobacter* spp.) by using API 20E biochemical systems and the oxidase test (Data not shown). FSM 321, 2.43, 2.80, and 2.82 were

Table 3. Chromogenic or fluorogenic reaction of 4 different media supplemented with α-glucosidase substrates in non-*E. sakazakii* (*Cronobacter* spp.)

Species	Strain No.	Source	Chromogenic or fluorogenic reaction			
			Oxoid	OK	VRBG	NGTE
E. hermanii	FSN 324	UGA	-	-	-	-
Salmonella spp.	1.11	FDA	-	-	-	-
E. cloacae	SP 118	NRC	-	-	-	-
E. coli	K12	FDA	-	-	-	-
E. vulneris	102-4a	NRC	+	+	+	-

UGA, Dr. Jeffrey Kornacki, University of Georgia, Athens, GA; FDA, Culture collection of U.S. Food and Drug Administration, College Park, MD; NRC, Dr. John Marugg, Nestle Research Center, Lausanne, Switwerland.

+, *E. sakazakii* (*Cronobacter* spp.); −, non-*E. sakazakii* (*Cronobacter* spp.).

Table 4. Sensitivity of 4 different media with supplemented with α-glucosidase substrates, 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside (BCIG) or 4-methyl-umbelliferyl α-D-glucoside (α-MUG), in 58 strains of *E. sakazakii* (*Cronobacter* spp.) at 37℃ for 24 h

Media	Total of *E. sakazakii*	Positive of *E. sakazakii*	Negative of *E. sakazakii*	Percentage of recovery (%)
Oxoid	58	56	2	96.6
OK	58	56	2	96.6
VRBG	58	56	2	96.6
NGTE	58	56	2	96.6

Percentage of recovery = [Positive / Total of *E. sakazakii* (*Cronobacter* spp.)] × 100.

Table 5. The comparison of colony size on the media with addition of various iron sources

	Oxoid	Oxoid2	Oxoid3	OK	C1	NGTE	NGTE8
Iron source	FAC	FE	FS	FC	X	FE	FS
Carbohydrate	X	X	X	X	Lac	Glu	Glu
α-Glucosidase substrates	Chro	Mug	Mug	Mug	Mug	Mug	Mug
E. sakazakii (*Cronobacter* spp.)	MC	MC	SC not grow well	MC	LC	LC	VSC not grow well
E. cloacae	LC	LC	No grow	MC	LC	LC	VSC
E. coli K12	LC	MC	No grow	MC	LC	MC	No grow
E. hermanii	MC	MC	MC	MC	MC	MC	SC
Salmonella spp.	MC (black)	MC (black)	MC (black)	MC (black)	MC	MC	No grow
E. vluneris	MC	SC	No grow	SC	MC	SC	No grow

Oxoid 2 and 3, substitution by α-MUG or Iron source in Oxoid.
NGTE 8, substitution by Iron source in NGTE.
FAC, ferric ammonium citrate; FE, ferrioxamine E; FS, ferrous sulphate; X, blank; Lac, Lactose; Glu, Glucose; MC, medium colony; LC, large colony; SC, small colony; VSC, very small colony.

determined by *E. sakazakii* (*Cronobacter* spp.) (Table 6).

To raise the sensitivity of *E. sakazakii* (*Cronobacter* spp.) in FSM 321, 2.43, 2.80, and 2.82, which showed partial positive or negative on 4 different media, we tried to compare the media with various iron source, FAC, FS, and FE, respectively (Data not shown). There is no significant difference in type of iron source (Table 7). However, media with FE was more ease to distinguish positive or negative under UV light because the intensity of fluorescence was stronger than media with FAC or FS (data not shown).

None of the non-*E. sakazakii* (*Cronobacter* spp.) strains showed fluorescent or chromogenic colonies on any of the media tested except *E. vulneris*, showing false

Table 6. Differences of strain identification using API 20E biochemical profiles in *E. sakazakii* (*Cronobacter* spp.) FSM 321, 2.43, 2.80, and 2.82, respectively

Species	Strain No.	Source	Oxidase	API20E (%)
E. sakazakii	FSM 321	NRC	-	*E. sakazakii* (59.5)
E. sakazakii	2.43	UGA	-	*E. sakazakii* (98.4)
E. sakazakii	2.80	UGA	-	*E. sakazakii* (98.4)
E. sakazakii	2.82	UGA	-	*E. sakazakii* (97.3)

NRC, Dr. John Marugg, Nestle Research Center, Lausanne, Switwerland; UGA, Dr. Jeffrey Kornacki, University of Georgia, Athens, GA.

positive on Oxoid, OK, and VRBG media and negative on NGTE medium (Table 3). It is assumed that the negative of *E. vulneris* was caused by glucose (Table 8).

Discussion

Various selective meida for *E. sakazakii* (*Cronobacter* spp.) have been developed by using of the fluorogenic or chromogenic enzyme substrates so as to eliminate the need for biochemical tests or subculture to confirm the identification of microorganisms [8-12,16,17]. Among the α-glucosidase substrates, 4-nitrophenyl-α-D- glucopyranoside and 4-mehtyl-umbelliferyl-α-glucoside were used as possible markers [18]. 4-nitrophenyl-

Table 7. The reaction of α-glucosidase substrates on various media with addition of various iron sources to detect *E. sakazakii* in FSM 321, 2.43, 2.80, and 2.82

	Oxoid	Oxoid1	Oxoid2	OK	VRBG	VRBG1	NGTE1	NGTE2	NGTE	NGTE3	NGTE4
Iron source	FAC	FAC	FE	FC	X	FE	X	FE	FE	FE	FAC
Carbohydrate	X	X	X	X	Lac	Lac	Glu	X	Glu	Lac	Glu
α-Glucosidase substrates	Chro	Mug	Mug	Mug	Mug	Mug	Mug	Mug	Mug	Mug	Mug
FSM321	−	−	−	−	−	+	+	−	+	+	+
2.43	−	+	+	−	−	+	+	+	+	+	+
2.80	+	+	+	+	+	−	−	+	−	−	−
2.82	+	+	+	+	+	+	−	+	−	+	−

Oxoid 1 and 2, substitution by α-MUG or iron source in Oxoid.
VRBG 1, substitution by iron source in VRBG.
NGTE 1, 2, 3, and 4: substitution by iron source or carbohydrate in NGTE.
FAC, ferric ammonium citrate; FC, ferric citrate; FE, ferrioxamine E.
Chro, 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside (BCIG).
Mug, 4-methyl-umbelliferyl α-D-glucoside (α-MUG).
X, blank; Lac, lactose; Glu, glucose.
+, positive of α-glucosidase substrates; −, negative of α-glucosidase substrates.

Table 8. The effective of various iron sources to remove false positive as *E. vulneris* on the media

	Oxoid	Oxoid1	Oxoid2	OK	VRBG	VRBG1	NGTE1	NGTE2	NGTE	NGTE3	NGTE4
Iron source	FAC	FAC	FE	FC	X	FE	X	FE	FE	FE	FAC
Carbohydrate	X	X	X	X	Lac	Lac	Glu	X	Glu	Lac	Glu
α-Glucosidase substrates	Chro	Mug	Mug	Mug	Mug	Mug	Mug	Mug	Mug	Mug	Mug
E. sakazakii (Cronobacter spp.)	+	+	+	+	+	+	+	+	+	+	+
E. cloacae	−	−	−	−	−	−	−	−	−	−	−
E. coli K12	−	−	−	−	−	−	−	−	−	−	−
E. hermanii	−	−	−	−	−	−	−	−	−	−	−
Salmonella	−	−	−	−	−	−	−	−	−	−	−
E. vluneris	+	+	+	+	+	+	−	+	−	+	−

Oxoid 1 and 2, substitution by α-MUG or Iron source in Oxoid.
VRBG 1, substitution by iron source in VRBG.
NGTE 1, 2, 3, and 4, substitution by iron source or carbohydrate in NGTE.
FAC, ferric ammonium citrate; FC, ferric citrate; FE, ferrioxamine E.
Chro, 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside (BCIG).
Mug, 4-methyl-umbelliferyl α-D-glucoside (α-MUG).
X, blank; Lac, lactose; Glu, glucose.
+, positive of α-glucosidase substrates; −, negative of α-glucosidase substrates.

α-D-glucopyranoside formed yellow-colored colonies, but has limitations because the yellow breakdown product, 4-nitrophenol, was easily diffusible on agar, making it difficult to read, but 4-mehtyl-umbelliferyl-α-glucoside produced distinct, not easily diffusible, and fluorescent colonies under UV irradiation.

Recently, Leuschener & Bew [10] combined the two characteristics of yellow pigmentation and enzymatic activity by supplementing nutrient agar with 4-mehtyl-umbelliferyl-α-glucoside. Isolates that are able to metabolize the substrate yield a yellow fluorescent product and can be identified as *E. sakazakii* (*Cronobacter* spp.). Also, the α-glucosidase hydrolyses the chromogenic substrate, 5-bromo-4-chloro-3-indoly α -D-glucopyranoside, producing blue-green colonies on this pale yellow medium. These two methods are a time-saving alternative to detect *E. sakazakii* (*Cronobacter* spp.). Unfortunately, the detection rate of *E. sakazakii* (*Cronobacter* spp.) did not reach 100%. Restaino et al. [13] recently developed R & F® *Enterobacter sakazakii* Chromogenic Plating Medium, which showed blue-black raised to domed colonies 1–2.0 mm diameter ± clear halos in *E. sakazakii* (*Cronobacter* spp.). But it needed 2nd step to inoculate R & F® *Enterobacter sakazakii* (*Cronobacter* spp.) screening biplating medium consisted of melibiose and sucrose so as to confirm *E. sakazakii* (*Cronobacter* spp.). Similar results were observed in this study showing the maximum detection rate of 96%. It appeared that at least two media should be combined to detect 100%.

Furthermore, previous studies fail to distinguish between *E. sakazakii* (*Cronobacter* spp.) and *E. vunleris* because both strains showed positive by using fluorogenic or chromogenic enzyme substrates [8,19]. *E. vulneris* is a gram-negative, oxidase-negative, fermentative, motile rod with the characteristics of the family *Enterobacteriaceae*, and two-thirds of the strains produced yellow pigment and no acid form D-sorbitol [19]. Farmer [20] reported that the false positive result with *E. vulneris* was expected since this species was reported to be 25% positive for α-glucosidase and 50% positive for yellow pigment production. Similarly, in our study showed that *E. vulneris* also utilizes α-glucosidase substrates resulting in false positives. Three previously developed media, Oxoid, OK, and VRBG, showed false positive for *E. vulneris*, but the NGTE media with glucose used in this study was negative for *E. vulneris*.

It is unclear but may be due to reason that *E. vulneris* was difficult to hydrolyse the fluorogenic or chromogenic enzyme substrates under the glucose-containing media [19]. Hence, *E. vulneris* did not produce the fluorescent colonies on media as negative reaction [19]. Since Butterworth et al. [21] reported that the difference of β-ribosidase activity was due to different rates of substrate uptake through the cell envelop or differences in the substrate affinity of the β-ribosidase enzyme within different species.

Lehner & Stephan [22] published that two different morphologies are exhibited by *E. sakazakii* (*Cronobacter* spp.) ATCC 29544 when grown on different plates: the rubbery colonies (Blood agar and Brain heart infusion agar) and the smooth colonies (Plate count agar), and also the intensity of the pigmentation may vary from stain to strain. Also this study demonstrated that the colonies formed on media of Oxoid and OX showed the rubbery, but the one of VRBG and NGTE showed smooth with slime (Data not shown).

It may due to complicated reaction between various iron sources and carbohydrates. In comparison of colony morphology, we observed that colonies of Ferrioxamine E containing media showed were smaller than those of FAC on *E. vulneris* strain, and also produced larger colony on *Enterobacter* species than on *Escheirchia* species and *Salmonella*. It may due to Ferrioxamine E, because it is utilized by *E. sakazakii* (*Cronobacter* spp.), but not by *E.vulneris* as *Escherichia* species. Also, FE could help not only distinguish *E. sakazakii* (*Cronobacter* spp.) from strains but also selective growth.

Conclusion

In conclusion, this study demonstrates the NGTE enabled not only detection of an equivalent number of positive colonies but also excluded false positives such as *E. vulneris* when compared with currently available media. We found out that only glucose could take important role of removal for *E. vulneris* as false positive on the media. After compared with the colony size and growth on the media, media with addition of FAC and FE revealed better than that of FS. In comparison of colony of *E. vulneris* strain, it was smaller on media with addition of FE than that of FAC. Ferrioxamine E containing media produced larger colonies in *Enterobacter* species than in *Escheirchia* species and *Salmonella*. Furthermore, FE containing media had strong intensity of fluorescence under UV light than other iron sources. Therefore, NGTE with FE, Glucose, and α-MUG showed the best result of detecting *E. sakazakii* (*Cronobacter* spp.) and of eliminating false positive as *E. vunleris* in this study. Further study is required to determine the mechanism between glucose and α-glucosidase enzyme of *E. vulneris*, and also to improve the recovery percentage of injured *E. sakazakii* (*Cronobacter* spp.) by using of various iron sources. Furthermore, we try to study the usability of FE to detect *E. sakazakii* (*Cronobacter* spp.) as selective growth factor.

Conflict of Interest

The authors declare no potential conflict of interest.

Acknowledgements

This paper was supported by the Konkuk University Researcher Fund, 2020. Also, the authors thank the support of all members of Center for One Health and Sensergen, too.

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