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Nurmi-type Culture Prepared using Culture Media without L-Cysteine Enhances *Salmonella* Exclusion in Hatched Layer Chicks

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To determine the influence of media composition on *Salmonella* exclusion of Nurmi-type cultures, two and four types of cultures in the first and second trial, respectively, were prepared from the cecal contents of conventional laying hens, and *Salmonella* exclusion was assessed in newly hatched chicks. In the first trial, modified Viande Levure (VL) broth or nutrient broth (NB) were used to prepare Nurmi-type cultures (N-VL and N-NB), which were administered to the newly hatched chicks. Twenty-four hours later, the chicks were challenged with *Salmonella enterica* Typhimurium EF85-9 (ST). ST recoveries ($\log_{10}$ colony forming units/g of cecal contents) from the N-VL-, N-NB-, and control-treated groups 5 days after the challenge were 7.6±0.6, 0.9±1.9, and 7.7±0.4, respectively. The results suggested the influence of L-cysteine (Cys) present in the VL broth. Thus, we determined the effect of Cys in the second trial. We prepared two other cultures using VL broth without Cys (N-VL−Cys) and NB with Cys (N-NB+Cys). ST recoveries from the cecal contents of the N-VL-, N-VL−Cys-, and control-treated groups were 6.3±0.9, 2.1±2.5, and 9.2±0.8, respectively. ST was not recovered from the N-NB- and N-NB+Cys-treated groups. To identify bacteria with *Salmonella* exclusion activity, we isolated 41 bacterial strains from the ceca of N-NB-treated chicks without *Salmonella* challenge. Most isolates were identified as *Enterococcus faecalis* or *E. mundtii* based on 16S rRNA gene sequencing, and only four cultures excluded *Salmonella*. Therefore, VL broth containing Cys was not always required for preparing Nurmi-type cultures. The use of media prepared with Cys at the lowest possible concentration or without Cys would promote to enhance *Salmonella* exclusion from Nurmi-type cultures.

Key words: competitive exclusion, *Enterococcus* sp., L-cysteine, Nurmi-type culture, PCR-DGGE, *Salmonella*

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Introduction

Nurmi and Rantala (1973) reported competitive exclusion (CE) of *Salmonella* infection in broiler chickens, and since then, CE cultures have been used worldwide for raising broiler and layer chickens as well as turkeys. Because of their immature immune systems, newly hatched chicks are readily infected by *Salmonella*, and infection can remain latent and subsequently become a serious disease in adult chickens, leading to food poisoning. Latent infection with *Salmonella* is a serious problem because the lack of symptoms allows the pathogen to spread horizontally through a poultry farm. Therefore, control of *Salmonella* infection in newly hatched chicks is very important.

Salmonella has been classified into >2500 serotypes according to the presence of O and H antigens (Grimont and Weill, 2007); however, vaccines effective against all strains are not available. Antibiotics may be used to control infection but cannot be administered to layer hens because the eggs will retain antibiotic residues. Therefore, the control of *Salmonella* infection in newly hatched chicks using a CE culture is a more appropriate strategy. In 1999, the European Union banned the addition of antibiotics to livestock feed as growth promoters. In 2000, experts from the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) assessed the risks caused by microbiological hazards in foods (FAO/WHO, 2000) and subsequently proposed that undefined CE products combined with other methods could effectively control *Salmonella* populations (FAO/WHO, 2009).

Pivnick and Nurmi (1982) reported that CE from defined bacterial cultures should be mixed with individual bacterial species cultured in suitable media rather than in Viande Levure (VL) broth, which is a traditional anaerobic medium. In contrast, Barrow and Tucker (1986) found that *Escherichia coli* cultured in nutrient broth (NB) exhibited *Salmonella* exclusion. Since that we focused on formulating a media

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composition for preparing Nurmi-type cultures. Here we evaluated *Salmonella* exclusion of two types of Nurmi-type cultures inoculated with conventional chicken cecal contents in media containing VL broth (modified by Barnes and Impey, 1970) or NB. We used bonito extract to prepare NB, because we considered using media prepared from bovines to remove an impaction of bovine spongiform encephalopathy. We then assessed the effect of each culture on *Salmonella* exclusion from chicken ceca and livers in an initial trial. The results indicated that L-cysteine (Cys) enhanced *Salmonella* exclusion. Therefore, we prepared four types of Nurmi-type cultures inoculated with conventional chicken cecal contents in VL broth or NB with or without Cys to assess their effects on *Salmonella* exclusion in chicken ceca contents and livers in a second trial. We used PCR-denaturing gradient gel electrophoresis (DGGE) to compare the bacterial flora present in these Nurmi-type cultures and attempted to isolate bacteria with *Salmonella* exclusion activity from the cecal contents of N-NB-treated chicks not challenged with *Salmonella*.

Materials and Methods

Analysis of Salmonella and Campylobacter in the CE Source

Five conventional layer hens were obtained from the Poultry Station at Sendai (Miyagi, Japan). Cecal contents were weighed and diluted 1:5 (w/v) with VL broth without Cys. The first dilutions (CE source) were exposed to O_2-free-CO_2 gas and stored at −80℃ until use. Each CE source was analyzed to detect the presence of *Salmonella* and *Campylobacter*.

For *Salmonella*, 1 m*l* of each CE source was inoculated in 9 m*l* of tetrathionate broth (TTB; Eiken Chemical, Tokyo, Japan), cultured at 41.5℃ for 24 h. After TTB was streaked on deoxycholate-hydrogen sulfide-lactose (DHL) agar plates (Nissui Pharmaceutical, Tokyo, Japan), it was further incubated for 7 days at room temperature. One ml of the culture was inoculated into 9 m*l* of fresh TTB, incubated at 37℃ for 24 h, and plated onto DHL agar. This procedure termed "delayed secondary enrichment" increased the sensitivity of *Salmonella* detection more than conventional culture method (Rigby and Pettit, 1980; Waltman *et al*., 1991). *Salmonella* infection of the liver was assayed using delayed secondary enrichment. Black colonies on the plates were analyzed using a *Salmonella* Nucleic Acid Test Kit (Fasmac, Kanagawa, Japan).

To determine the presence of *Campylobacter*, each CE source was cultured in Preston *Campylobacter*-selective medium (Oxoid, Hampshire, UK), and the cultures were spread on Preston *Campylobacter*-selective agar plates under microaerobic conditions using an AnaeroPack MicroAero (Mitsubishi Gas Chemical, Tokyo, Japan). The colonies were analyzed using a *Campylobacter* test kit (Meiji Seika, Tokyo, Japan).

Each CE source was analyzed for the presence of lecithinase-positive *Clostridium perfringens* colonies on Neomycin Nagler agar plates (Lowbury and Lilly, 1955). Four CE sources with undetectable numbers of *C. perfringens* were mixed in equal volumes, and Nurmi-type cultures were prepared after confirming that *Salmonella* and *Campylobacter* were undetectable.

Preparation of Nurmi-type Cultures

VL broth or NB was used as the basal culture medium for preparing Nurmi-type cultures. VL broth contained 10 g Bacto Tryptone (BD Difco), 3 g Lab-Lemco powder (Oxoid), 5 g Bacto Yeast Extract (BD Difco), 2.5 g glucose, 5 g NaCl, 0.4 g L-cysteine HCl (Kanto Chemical Co., Inc., Tokyo, Japan), and 0.6 g agar in 1 liter of distilled water adjusted to pH 7.2 using 1 N NaOH (Barnes and Impey, 1970). NB contained 10 g Bonito extract (Wako Pure Chemical Industries, Ltd., Tokyo, Japan), 10 g peptone (Wako), 2 g NaCl, 5 g K_2HPO_4, and 0.8 g agar (Wako) in 1 liter of distilled water adjusted to pH 7.0 using a 1 N NaOH solution. Other broth cultures were prepared with or without Cys. Cys was added to NB (NB＋Cys) and not to VL broth, which usually contains Cys in its original formulation (VL−Cys). The final Cys concentration in each broth was 2 mM (Barnes and Impey, 1970). Each Nurmi-type culture was started by inoculating 1 m*l* of mixed CE source into 100 m*l* of each medium followed by incubation at 37℃ for 24 h under anaerobic conditions without agitation, using the AnaeroPak method (Mitsubishi Gas).

Salmonella Strain

Nalidixic acid (NA)-resistant *Salmonella enterica* Typhimurium EF85-9 (ST) was originally isolated from a patient with food poisoning in Tokyo, and this strain was obtained from Dr. Takeshi Ito at the Tokyo Metropolitan Research Laboratory of Public Health. When preparing test cultures, an aliquot of the stock culture was spread on a soybean-casein digest (SCD) agar plate (Nissui) and incubated at 37℃ overnight. Cells from a single colony were inoculated into SCD broth (Nissui), incubated at 37℃ for 20 h, and 1 m*l* of the culture was used to challenge one chick. *Salmonella* were detected by counting H_2S-containing black colonies on DHL agar plates containing 20 μg/m*l* NA (DHL-NA).

Chickens

Thirty newly hatched (0 day old) White Leghorn (Julia) male chicks were included in the first trial, and 50 other 0-day-old White Leghorn (Julia) male chicks were included in the second. All chicks were obtained from a commercial hatchery (Koiwai Farm Ltd., Iwate, Japan). All experiments were conducted in accordance with the policies of the Animal Experiments Committee of Akita Prefectural University.

Salmonella Challenge Experiments

First Trial. To determine *Salmonella* exclusion mediated by two types of Nurmi-type cultures, challenge experiments were conducted according to an assay recommended to evaluate different CE preparations (Mead *et al*., 1989). Thirty newly hatched chicks were divided into groups as follows: Group 1 was treated with a sterile physiological saline solution. Group 2 was treated with a Nurmi-type VL (N-VL) culture. Group 3 was treated with a Nurmi-type NB (N-NB) culture. One day after transport to the laboratory, the 0-day-old chicks were administered 1 m*l* of Nurmi-type culture directly into the crop using a cannula. Twenty-four hours

after treatment, the chicks were challenged with approximately 1×10^3 colony forming units (cfu) of ST applied directly into the crop using a cannula (day 2). Feed and water were then provided *ad libitum*. Five days after ST challenge (day 7), all chicks were anesthetized using diethylether and sacrificed. After opening the abdominal cavity, the ceca and liver were removed aseptically and transferred to a sterile petri dish. ST recoveries from each chick's cecal content and liver culture were measured.

Second Trial. To evaluate *Salmonella* exclusion in the first trial and to determine the effects of the media additives, four types of Nurmi-type cultures were prepared, and *Salmonella* challenge experiments were conducted according to the schedules of the first trial. Fifty newly hatched chicks were divided into the groups as follows: Group 1 was treated with a sterile physiological saline solution. Group 2 was treated with N-VL. Group 3 was treated with N-VL without Cys (N-VL−Cys). Group 4 was treated with N-NB with Cys (N-NB +Cys). Group 5 treated with N-NB. The VL broth and NB +Cys broth each contained 2 mM Cys.

Salmonella Recovery from Cecal Content and Liver

Cecal contents were weighed, diluted 10-fold (w/v) with selenite cystine broth (Nissui), and serially diluted 10-fold with sterile saline to a final dilution of 10^{-6}. The first dilution (0.5 m*l*) was spread onto two plates of DHL-NA, and 0.1 m*l* each of the other dilutions were spread onto DHL-NA plates. The number of H_2S-producing black ST colonies after incubation for 24 h and 48 h at 37℃ was determined, and ST recovery was calculated and is expressed as the $\log_{10}$ cfu/g of intestinal contents±standard deviation (SD). The presence of black colonies in the streak indicated infection with ST.

To evaluate the invasion of the chicks by *Salmonella* through the alimentary canal, livers were subjected to delayed secondary enrichment and were assayed for *Salmonella* colonies. Briefly, approximately 1 g of the left lobe of each liver was cut into pieces using sterilized scissors and diluted approximately 10-fold (w/v) in selenite cystine broth, incubated for 18 h at 37℃, and spread onto DHL-NA plates. To increase sensitivity of ST detection, the remainder of the selenite cystine broth was subjected to delayed secondary enrichment, the cultures were spread on DHL-NA plates, and incubated for 18 h at 37℃.

Statistical Analysis

Statistical analysis of *Salmonella* recovery after the first trial and the differences in the numbers of bacteria between control and each treatment were analyzed using the *t*-test. The data from the second trial between treatments was performed using one-way analysis of variance (ANOVA) and Scheffe's test for post-hoc comparisons. The analyses were performed using KaleidaGraph version 4.1 (Synergy Software). Statistical significance was defined as $P<0.05$.

PCR-DGGE Analysis

Bacterial genomic DNA from Nurmi-type cultures was extracted using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, except for lysis at 90℃. PCR was performed using 2 × *GoTaq* Green Master Mix (GMM; Promega, Madison, WI, USA). Two primers were used, GC-357F (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCCAG-3′) and 518R (5′-GTATTACCGCGGCTGCTGG-3′). Two bases were added to the 5′ end of the 518R primer as described by Muyzer *et al*. (1993). The PCR mixture (20 μl) contained 10 μl of GMM, 2 μl of each primer (20 mM), 50 ng of template DNA, and Milli-Q water as required. Thermal cycling conditions followed those of Muyzer *et al*. (1993). DGGE gels were prepared using 30%−60% linear denaturing gradients in 8% polyacrylamide according to the DCode System instruction manual (Bio-Rad Laboratories Inc., Hercules, CA, USA). DGGE was performed using a constant voltage of 130 V for 5 h at 60℃ in TAE buffer (40 mM Tris-HCl, 40 mM acetic acid, 1 mM EDTA). After electrophoresis, the gel was stained with SYBR Gold (Molecular Probes, Life Technologies, Eugene, OR, USA), and the bands were visualized using a Luminescent Image Analyzer LAS-1000 (Fuji Film, Tokyo, Japan). Major bands were excised from the gel using a sterile blade, purified with the Wizard SV Gel and PCR Clean-up System (Promega), and PCR-amplified with the 357F (5′-CCTACGGGAGGCAGCCAG-3′) and 518R primer pair. After TA cloning using a pGEM T-vector system (Promega), the plasmid with PCR-amplified fragments were used to transform *E. coli* DH5α, and the DNA sequences of each plasmid insert were determined in the 5′ and 3′ directions with the M13 primers RV (5′-CAGGAAACAGCTATGAC-3′) and M4 (5′-GTTTTCCCAGTCACGAC-3′) as described in the supplier's instruction manual. Bacteria that were the most closely related to the sampled DNAs were identified using BLASTN program (https://blast.ncbi.nlm.nih.gov/Blast.cgi; BLAST2. 2. 29+; Zhang *et al*., 2000) with 16S ribosomal RNA sequences database for Bacteria and Archaea (Morgulis *et al*., 2008). DNA sequences of DGGE bands were deposited in the DNA Databank of Japan (DDBJ) under accession numbers AB899845–AB899857.

Isolation of Bacteria from Cecal Contents of Hatched Chicks Treated with N-NB and Evaluation of Salmonella Exclusion

We attempted to isolate bacteria from the cecal contents of chicks administered N-NB on day 3 without ST challenge. Cecal contents were diluted under anaerobic condition by spraying with O_2-free-CO_2 gas, and the dilutions were spread on Bacteroides agar plates (Nissui) and cultured at 37℃ for 18 h under anaerobic conditions with an AnaeroPak. Each bacterial culture with NB was evaluated in triplicate for *Salmonella* exclusion in hatched chicks. The trials were conducted at the Narita Animal Science (NAS) Laboratory Co. (Chiba, Japan) from August 2008 to March 2009 and from October 2009 to March 2010. Each of five newly hatched White Leghorn (Julia) male chicks per bacterial culture was used in experiments 1 and 2, and each of five newly hatched White Leghorn (Julia) female chicks per culture was used in experiment 3. CE Tect (commercial CE; Scientific Feed Laboratory Inc., Tokyo, Japan) was treated with 1 m*l* of a 1:25 dilution per chick.

Isolates were identified using BLAST to search the Ribosomal Database Project (http://rdp.cme.msu.edu/classifier/classifier.jsp) for sequences with ＞99% identity to the 16S rRNA gene using the merged DNA sequences determined with the primers 27F (5′-GTTTGATCCTGGCTCAG-3′), 519F (5′-CAGCMGCCGCGGTAATWC-3′), 806R (5′-CTACCAGGGTATCTAATC-3′), and 1492R (5′-TACCTTGTTACGACTT-3′) (Kane *et al*., 1993; Paster *et al*., 1994; Suzuki and Giovannino, 1996). Two isolated strains designated APU-14 and APU-27 were also sequenced using primers 518R and 1114F (5′-GCAACGAGCGCAACCC-3′; Lane, 1991). The data were deposited in the DDBJ under accession numbers AB898299–AB898340, and the strains were archived at the National Institute of Technology and Evaluation (NITE) and designated P-1528 and P-1529.

Results

Salmonella Challenge

The results of the two ST challenge experiments using newly hatched chicks are shown in Table 1. In the first trial, ST recoveries ($\log_{10}$ cfu/g±SD) from the cecal contents of the N-VL-, N-NB-, and control-treated groups were 7.6± 0.6, 0.9±1.9, and 7.7±0.4, respectively, and the recovery from liver cultures after delayed secondary enrichment were 67%, 60%, and 80%, respectively. ST recovery from the cecal contents of the N-NB-treated group was significantly decreased ($P<0.01$) compared with the N-VL- and control-treated groups.

In the second trial, ST recovery ($\log_{10}$ cfu/g±SD) from the cecal contents of the N-VL-, N-VL－Cys-, and control-treated groups were 9.2±0.8, 6.3±0.9, and 2.1±2.5, respectively. In contrast, ST colonies in the cecal contents were not detected in the N-NB- and N-NB＋Cys-treated groups. There were significant differences between the treated groups ($P<0.05$). Further, the recoveries from the liver culture after the delayed secondary enrichment of the N-VL-, N-VL－Cys-, N-NB-, N-NB＋Cys-, and control-treated groups were 30%, 0%, 0%, 30%, and 60%, respectively. In particular, ST was not detected in the liver even for the Nurmi-type cultures prepared without Cys (N-VL－Cys and N-NB) after delayed secondary enrichment culture using selenite broth.

PCR-DGGE Analysis

We performed PCR-DGGE analysis to identify the major bacteria in the four types of Nurmi cultures after the second trial (Fig. 1), and the most closed related bacterial species are shown in Table 2. In each of the N-VLs-treated groups, the bands corresponded to *Enterobacteriaceae*, *C. perfringens*, *Lactobacillus* sp., and *L. reuteri*. In each of the N-NBs-treated groups, the bands corresponded to *Bacteroides* sp., *Fusobacterium* sp., and *Pseudoflavonifractor capillosus*.

Isolation of Bacteria and Evaluation of Salmonella Exclusion

The 41 colonies isolated from Bacteroides agar plates were initially identified as *Bacteroidaceae* according to the PCR-DGGE results (Table 2), by our previous analysis of the bacterial flora of commercial CE preparations and the intestinal contents of commercial CE-treated chicks using a culture method (unpublished data). However, 16S rRNA gene sequencing identified most colonies as *Enterococcus faecalis* or *Enterococcus mundtii*. *Salmonella* exclusion activity of isolated bacteria was evaluated with newly hatched White Leghorn (Julia) males (experiments 1 and 2) and females (experiment 3). Sixteen isolates were tested in experiment 1, and 25 isolates were tested in experiment 2. Thirteen isolates selected from experiments 1 and 2 were tested in experiment 3. The results of experiment 3 are shown in Table 3. ST recoveries ($\log_{10}$ cfu/g) from the ceca

Table 1. ***Salmonella*** **recovery from chicks treated Nurmi-type cultures**

Nurmi-type culture administrated	Chicks n	*Salmonella* counts: Cecum $\log_{10}$ cfu/g±SD	*Salmonella* counts: Liver Positive/total (%)*[1]
1st trial			
None	10	7.7 ± 0.4^{A}	8/10 (80)
N-VL	9*[2]	7.6 ± 0.6^{A}	6/ 9 (67)
N-NB	10	0.9 ± 1.9^{B}	6/10 (60)
2nd trial			
None	10	9.2 ± 0.8^{a}	6/10 (60)
N-VL	10	6.3 ± 0.9^{b}	3/10 (30)
N-VL－Cys	10	2.1 ± 2.5^{c}	0/10 (0)
N-NB	10	N. D. ^{d}	0/10 (0)
N-NB＋Cys	10	N. D. ^{d}	3/10 (30)

In the 1st trial, Means with different superscript letters (A and B) are significantly differences by *t*-test ($P<0.01$).
In the 2nd trial, Means with different superscript letters (a, b, c and d) are significantly differences by one-way ANOVA and Scheffe's test for post hoc comparisons ($P<0.05$).
N. D.＝not detected; it was treated as zero formally in statistical analysis.
*[1] Number of chick detected *Salmonella* colonies after delayed secondary enrichment.
*[2] One chick was lost during a period after *Salmonella* challenge.

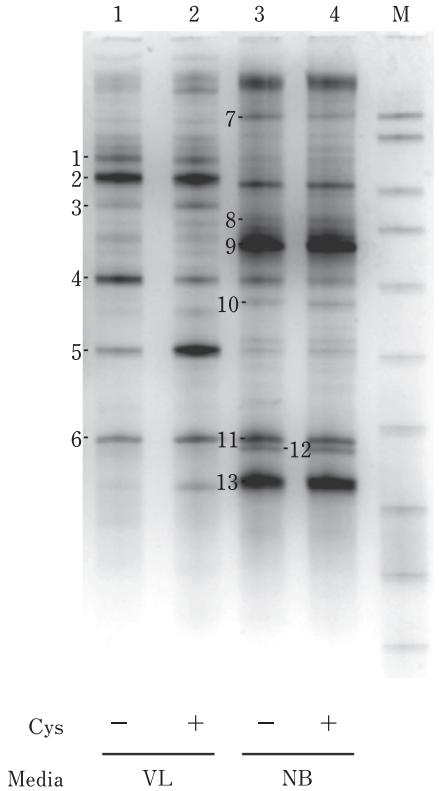

Fig. 1. **PCR-DGGE analysis of bacterial flora present in custom-made Nurmi-type cultures.** *Lane 1*, Nurmi-type culture prepared using VL broth without L-cysteine (N-VL−Cys); *Lane 2*, Nurmi-type culture prepared using VL broth (N-VL); *Lane 3*, Nurmi-type culture prepared using NB (N-NB); *Lane 4*, Nurmi-type culture prepared using NB with L-cysteine (N-NB+Cys); *Lane M*, DGGE marker II (Nippon Gene, Tokyo, Japan). The numbers on the gel indicate bands that were subjected to DNA sequence analysis (Table 2). The gel image was converted to black and white.

of the groups treated with APU-2, -14, -27, and -28 were 5.0 ±2.2, 2.5±2.5, 5.1±2.1, and 5.7±1.3, respectively, and recoveries were significantly lower for the control-treated group (7.6±0.9) ($P<0.01$ or $P<0.05$). ST recovery from the APU-14-treated group was lower than that of commercial CE (5.0±1.4), and the recoveries from the APU-2, -27, and -28 groups were not significantly different than those of the commercial CE preparation.

Discussion

Here we studied the effects of media composition and additives on *Salmonella* exclusion in Nurmi-type cultures for the following reasons: first, *E. coli* isolated from sewage and from an abattoir drain following culture in NB exhibits CE (Barrow and Tucker, 1986). Second, to address problems caused by bovine spongiform encephalopathy, we determined the effects of change in media composition made from bovine to other types for Nurmi-type culture preparation. Therefore, we used bonito extract to prepare NB. We compared *Salmonella* exclusion using Nurmi-type cultures prepared from VL broth and NB. We further evaluated *Salmonella* exclusion in cultures supplemented with Cys (N-VL, N-VL−Cys, N-NB, and N-NB+Cys).

After the first trial, the N-NB-treated group was more effective for *Salmonella* exclusion than the N-VL-treated group (Table 1). We next focused on the media additive L-cysteine and evaluated VL broth without Cys and NB with Cys for *Salmonella* exclusion using four Nurmi-type cultures in the second trial. The N-NB-treated group was more effective for *Salmonella* exclusion than the N-VL-treated group in the first and second trials. In both trials, the N-NB-treated group exhibited lower ST recovery from intestinal contents than the N-VL-treated group. In the N-NB-treated group, there were no detectable ST colonies in the ceca, and ST liver invasion was not detected even after delayed secondary enrichment (Table 1). VL medium is used for the anaerobic culture of normal gut contents. In contrast, in defined bacterial cultures, each bacterium is cultured in its own

Table 2. **DNA Sequence analysis of PCR-DGGE-generated bands from samples shown in Fig. 1**

Band no.	Accession number	Length (bp)	Closest relative	% Identity
1	AB899845	161	*Enterococcus cecorum*	98
2	AB899846	160	*Lactobacillus* sp.	92
3	AB899847	160	Enterobacteriaceae	100
4	AB899848	160	Enterobacteriaceae	100
5	AB899849	135	*Clostridium perfringens*	99
6	AB899850	160	*Lactobacillus reuteri*	99
7	AB899851	155	*Bacteroides* sp.	92
8	AB899852	139	*Fusobacterium mortiferum*	100
9	AB899853	139	*F. mortiferum*	100
10	AB899854	135	*C. perfringens*	100
11	AB899855	160	Enterobacteriaceae	100
12	AB899856	155	*Bacteroides* sp.	90
13	AB899857	138	*Pseudoflavonifractor capillosus*	99

Table 3. ***Salmonella*** **exclusion effect by single bacterial culture for female hatched chicks (Julia) at experiment 3**

Treatment	ID[a]	Chicks (n)	Administrated bacterium[b]	Challenged ST[b]	ST recovery $\log_{10}$ cfu/g±SD	Av. gain g±SD	Feed intake
None		5	—	2.7	7.6±0.9	23.8±2.0	2.49
APU- 2[c]	Em	5	8.3	2.7	5.0±2.2*	28.4±2.9*	1.92
- 3[c]	Em	5	8.1	2.7	8.3±1.0	26.9±6.7	2.26
-11[c]	Em	5	9.1	2.7	8.3±0.2	17.2±7.9	3.36
-14[c]	Ef	5	8.6	2.7	2.5±2.5**	25.2±4.4	2.10
-21[d]	Em	5	9.0	2.7	7.3±0.7	27.2±4.5	2.09
-23[d]	Em	5	8.7	2.7	8.0±0.3	29.3±6.4	1.99
-24[d]	Em	5	8.8	2.7	9.4±0.7	29.2±5.0*	1.95
-27[d]	Em	5	8.7	2.7	5.1±2.1*	26.5±6.6	2.13
-28[d]	Em	5	8.5	2.7	5.7±1.3*	23.5±7.2	2.28
-29[d]	Em	5	8.7	2.7	8.7±0.2	22.0±5.1	2.79
-32[d]	Em	5	8.7	2.7	8.4±0.1	29.6±3.7**	2.10
-39[d]	Em	5	8.8	2.7	8.4±0.3	29.0±5.1*	2.08
-41[d]	Ef	5	8.5	2.7	8.1±0.2	27.3±5.2	2.03
Commercial CE		5		2.7	5.0±1.4**	35.2±3.4**	1.98

Bacteria isolated from cecal contents of 3-d-old chicks treated by Nurmi-type culture prepared with nutrient broth without *Salmonella* challenge. The isolated were selected from *Salmonella* challenge experiment 1 and 2.
[a], Bacteria species identified by BLAST search with 16S rRNA gene sequence; Ef, *E. faecalis*; Em, *E. mundtii*; [b], $\log_{10}$ cfu /a chick; [c], bacteria were selected at experiment 1; [d], bacteria were selected at experiment 2; -, no administration; empty column, unknown; *, **, significant difference against none treated group, $P<0.05$, $P<0.01$, respectively.

optimum media and then the bacteria are mixed (Pivnick and Nurmi, 1982). Although we selected VL broth and NB, each Nurmi-type culture was prepared with no agitation under anaerobic conditions after inoculation with cecal contents, and we found that media without Cys were very effective for the enhancement of Nurmi-type cultures.

The assessment of the effect of CE conducted by Pivnick and Nurmi (1982) led them to propose that the number of *Salmonella* colonies per gram of gut content was useful for assessing *Salmonella* infection. To standardize ST challenges, Mead *et al*. (1989) termed this value the "infection factor" (IF) and introduced another criterion calculated by dividing the IF value of the untreated group by that of the CE product-treated group, which they designated the "protection factor" (PF). Mead *et al*., suggested that cultures with PF values <4 would be insufficient to mitigate *Salmonella* infection in commercial poultry flocks. According to the results of the second trial conducted here, the mean *Salmonella* recovery ($\log_{10}$ cfu/g) from the control group was 9.2 (i.e., the IF value). Therefore, the PF values for N-VL, N-VL−Cys, N-NB, and N-NB+Cys were calculated 1.4, 4.4, >4, and >4, respectively. The values of N-VL−Cys, N-NB, and N-NB+Cys may be similar to those determined in a defined mixture of 32 bacterial species (Schneitz and Hakkinen, 1998).

In the second trial, ST was undetectable in cecal contents from groups administered the Nurmi-type cultures prepared using N-NB and N-NB+Cys. Further, ST was not recovered from the liver cultures of groups administered N-VL−Cys or N-NB. To provide an explanation for these results, we conducted PCR-DGGE analysis to identify the bacterial flora present in the Nurmi-type cultures (Fig. 1 and Table 2), and we attempted to isolate bacteria with *Salmonella* exclusion activity from the cecal contents of N-NB-treated chicks that were not challenged with *Salmonella*. However, the number of DGGE bands observed here was clearly lesser than that reported by Waters *et al*. (2006), who characterized bacterial flora in 10 batches of prototype Nurmi-type cultures using VL broth. This might be explained by the genomic DNA extraction method or the selection of PCR primers. The bands corresponding to *Lactobacillus* sp. and *L. reuteri* were found in only the N-VL cultures with or without Cys.

Certain *Lactobacillus* sp. are used in commercially available defined bacterial cultures (Tellez *et al*., 2012), and *L. reuteri* has probiotic potential for treating chicks with *Salmonella* infections (Zhang *et al*., 2012). Further, the co-culture of *L. reuteri* and *E. coli* is affected by the production of the bacteriocin reuterin (Schaefer *et al*., 2010). Although we did not determine the presence of bacteriocins such as reuterin here, bacteriocins might explain why *Salmonella* exclusion of the N-VL−Cys-treated group was greater than that of the N-VL-treated group. However, *Salmonella* exclusion was greater in the groups treated with the Nurmi-type cultures prepared without Cys (N-VL−Cys and N-NB) compared with those containing Cys (N-VL and N-NB+Cys). These findings suggest that the influence of the Cys concentration Nurmi-type cultures should be re-evaluated.

Lactic acid bacteria such as *Lactobacillus* sp. may grow relatively well in VL, and bacteria such as *Bacteroides* sp. may grow well in NB. The CE concept was applied to other bacterial species such as *Campylobacter* sp. (Aho *et al*., 1992; Schoeni and Doyle, 1992), *C. perfringens* (Fukata *et al*., 1991; Hofacre *et al*., 2002), *Listeria monocytogenes* (Hume *et al*., 1998), and pathogenic *E. coli* (Stavric *et al*.,

1992; Hofacre *et al*., 2002) as well as *Salmonella*. However, this does not necessarily indicate that anaerobic culture media are the best choice for Nurmi-type culture preparations in all cases. In the two N-NB cultures, we detected bands corresponding to anaerobic bacteria (*Bacteroides* sp., *Fusobacterium* sp., and *P. capillosus*). *Bacteroides capillosus* is now assigned to *P. capillosus* (Carlier *at al*., 2010), and *Bacteroides* sp. have been included as a mixture prepared from defined bacteria (Impey *et al*., 1982).

According to the results of PCR-DGGE analysis presented here and the increased prevalence of Bacteroidaceae in commercial CE-treated chicks using a culture method (unpublished data), we used Bacteroides selective agar for isolating bacteria. However, 16S rRNA gene sequencing identified most isolates as *E. faecalis* or *E. mundtii*. Although these bacteria are present in defined cultures (Scanlan, 1997; Wagner *et al*., 2002), to the best of our knowledge, there are no reports of *Salmonella* exclusion in chicks by a culture containing a single bacterial species. This may explain why N-NBs-treated chicks exhibited higher *Salmonella* exclusion compared with those treated with N-VLs.

In summary, we prepared Nurmi-type cultures using either VL or NB as the base medium and assessed the response of newly hatched chicks treated with each to a *Salmonella* challenge. The data led us to conclude that anaerobic culture media do not always require Nurmi-type culture preparation and that media without Cys may enhance *Salmonella* exclusion. Further, we also propose that changing the origin of media components improves the exclusion activity of Nurmi-type culture.

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