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Probiotic *Limosilactobacillus reuteri* KUB-AC5 inhibits growth of clinical *Salmonella enterica* typhimurium isolates

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Abstract

Salmonella enterica Typhimurium (STM) is a causative agent of an important foodborne disease, non-typhoidal salmonellosis (NTS). Although NTS causes self-limiting acute diarrhea in a healthy host, it can result in a life-threatening invasive NTS in immunocompromised hosts. An increase in multi-drug resistant (MDR) STM strains has been a major concern in the healthcare system and a search for alternative treatments has become imperative. Several probiotic strains have been extensively studied for their role against *Salmonella*. The Gram-positive bacterium *Limosilactobacillus (Lactobacillus) reuteri* KUB-AC5 (AC5) isolated from chicken gut has demonstrated an antagonistic effect to STM. However, the differences in STM strains could result in different outcomes of the AC5 intervention. In this study, we investigated the growth inhibitory effect of AC5 on five randomly selected MDR clinical STM strains (STMC) isolated from acute NTS patients admitted to Maharaj Nakorn Chiang Mai Hospital. All five STMCs (SMTC53, 78, 81, 101, and 107) exhibited MDR phenotypes. By using agar spot, cell-free supernatant diffusion agar and liquid medium co-culture assay, we found that AC5 can significantly inhibit the growth of all STMCs. Our study suggested that AC5 has the potential to be a probiotic intervention against STM infection in Thailand.

Keywords: Non-typhoidal *Salmonella*, Probiotic *Limosilactobacillus (Lactobacillus) reuteri* KUB-AC5, Antagonistic effect, Clinically isolated *Salmonella*, Multi-drug resistant *Salmonella*

1. Introduction

Non-typhoidal salmonellosis (NTS) is an important foodborne disease worldwide, including Thailand, caused by the ingestion of Gram-negative bacterium *Salmonella enterica* serovars [1,2]. An acute NTS in a healthy host is usually self-limiting. However, life-threatening NTS, or invasive NTS, could develop in immunocompromised hosts [3,4]. Two important serovars of *Salmonella* that infect human are the serovars Typhimurium and Enteritidis. Increasing antibiotic resistance in non-typhoidal *Salmonella* strains is a major problem in public health, agriculture, and food industry [5,6]. Misuse and overuse of antibiotics play an important role in the significant increase of multi-drug resistant (MDR) *Salmonella* strains [7,8]. Hence, alternatives to antibiotics against NTS are urgently needed [9].

Probiotics are living microorganisms that benefit host health when administered at an optimal dose and appropriate duration [10]. *Limosilactobacillus (Lactobacillus)* is a Gram-positive bacterium that has been extensively used as a probiotic against several pathogens including *Salmonella* [11-13]. *Limosilactobacillus (Lactobacillus) reuteri* has been shown to activate macrophage phagocytic activity against *Salmonella* invasion in mice [14]. However, inconsistent results regarding the efficacy of probiotic *L. reuteri* against *Salmonella* have been reported. The strain-specific effect of probiotics plays a major role in the reported inconsistent outcomes. Mechanisms exerted by probiotic bacteria in the protection against gastrointestinal tract (GI) infection can be divided into two main groups [15,16]. First, the probiotic bacteria directly inhibit the growth of pathogens by competing for nutrients and space, and secretion of antimicrobial substances such as bacteriocins or toxins to kill the pathogen [17,18]. Second, probiotic bacteria indirectly manipulate the gut immune response to subsequently enhance the protective functions of the gut towards pathogens. For example, *L. reuteri* increases production of secretory IgA and gut epithelium-derived antimicrobial peptides [19,20].

L. reuteri KUB AC-5 (AC5) was first isolated from chicken intestine [21]. Since its isolation, several studies into its antagonistic effect to various kinds of pathogenic microorganisms have been tested both *in vitro* and *in vivo* [21-23]. Recently, Nakphaichit and team showed that AC5 can reduce the colonization of *S.* Enteritidis in broiler chickens [23]. The authors found that low and high doses (10^5 and 10^7 colony-forming units (CFU), respectively) of AC5 can eliminate *S.* Enteritidis in chicken ileum and cecum. Moreover, a high dose of AC5 induces changes in the microbiota population of the gut, increasing levels in *Lactobacillaceae* being found in both the ileum and cecum of broiler chicken.

S. Typhimurium (STM) is a major cause of acute NTS in human and warm-blooded animals [24]. The difference in levels of pathogenicity of *Salmonella* is due to its differences in host range and strains which play a crucial role in NTS pathogenesis [25]. Clinical *Salmonella enterica* Typhimurium (STMC) isolates from Thai NTS patients caused less pathogenicity in a mouse colitis model of *Salmonella* infection than it did in humans, those strains exhibiting high virulence in human colonic epithelium invasion assay [26]. In this study we investigated STMC growth inhibition of AC5 using agar diffusion and growth assays.

2. Material and methods

2.1 Antibiotic susceptibility test of clinically isolated Salmonella

Antibiotic susceptibility was tested using a disk diffusion method following the M100 protocol of the Clinical and Laboratory Standards Institute (CLSI) [27]. Briefly, two to three fresh colonies were resuspended in 3 mL phosphate buffer saline (PBS) and standardized to the turbidity equivalent of a 0.5 McFarland standard. The bacterial inoculum was spread over the entire agar surface of Mueller Hinton (MH; Oxoid, U) agar plates. Then, the antibiotic disks were placed on the surface and the plates were incubated at 37°C for 16-18 h. The diameter of the zone of inhibition was measured in millimeters using sliding calipers. The interpretation was based on the CLSI guidelines. The following antibiotic (Oxoid, Basingstoke, UK) and disk potencies (µg of each antibiotic) were used: amoxicillin/clavulanic acid (30), aztreonam (30), cefepime (30), cefotaxime (30), cefoxitin (30), ceftazidime (30), ceftriaxone (30), cefuroxime (30), cephazolin (30), doxycycline (30), levofloxacin (5), nalidixic acid (30), streptomycin (10), and tetracycline (30). A reference strain *Escherichia coli* ATCC 25922 was used as a quality control.

2.2 Bacterial strains and culture conditions

L. reuteri KUB-AC5 isolated the from chicken intestine was obtained from Assoc. Prof. Sunee Nitisinprasert, Kasetsart University, Bangkok, Thailand. Five clinical strains of *S.* Typhimurium (STMC53, 78, 81, 101, and 107) were isolated from stool bacterial cultures from acute gastroenteritis patients admitted to Maharaj Nakorn Chiang Mai Hospital, Chiang Mai, Thailand. AC5 was grown in de Man, Rogosa, and Sharpe (MRS) broth and STMC isolates were grown in Luria-Bertani (LB) broth at 37°C. Culturing was carried out for 16-18 h and accompanied by shaking.

2.3 Anti-Salmonella activity assay

The antagonistic effect of AC5 against *S.* Typhimurium was determined by agar spot assay. In brief, 20 µL of an overnight culture of AC5 was dropped on MRS agar plates and incubated at 37°C for 18 h to allow spots to develop. Then, 200 µL of approximately 10^{10} CFU/mL overnight cultures of each *Salmonella* strain was mixed into LB soft agar (0.7% agar) and poured over the plate.

The antimicrobial effects of the cell-free supernatant (CFS) were determined by agar well diffusion assay. AC5 CFS was *separated* by centrifugation (5000 g, 10 min) and filtered using 0.22 µm sterile filters. The overnight culture of each *Salmonella* strain was mixed into LB soft agar (0.7%) and poured onto the plates. The CFS (60

μL) was added into the 6-mm diameter wells bored in solidified plates. All plates were incubated at 37°C for 18 h and the zones of inhibition were measured. A clear zone of more than 1 mm around the spot was considered as positive [28].

2.4 Growth assays of L. reuteri KUB-AC5 and Salmonella

Growth assay of *Limosilactobacillus* (*Lactobacillus*) and *Salmonella* were done as previously described [29]. In brief, 100 μL of *L. reuteri* KUB-AC5 or *S.* Typhimurium subcultures was inoculated separately in 10 mL co-culture medium for the monoculture assay. The co-culture assay was performed by adding 100 μL of the 1:1 mixture of AC5 and STMCs into 10 mL co-culture medium. Then, all growth assays were carried out at 37°C without shaking and CFU/mL were calculated at the indicated time points. The CFU in AC5 and STMC were determined using a serial dilution technique using MRS agar plates with kanamycin (for AC5) and LB agar plates with streptomycin and tetracycline (for STMCs). Plates were then incubated at 37ºC for 18 h and the CFU were determined.

3. Results

Five clinical strains of *S.* Typhimurium (STMC53, 78, 81, 101, and 107) were tested to identify antibiotic resistant phenotypes as shown in Table 1. All strains showed a MDR (more than three drugs) phenotype. Only STMC78 was resistant to cefoxitin and only STMC107 was resistant to nalidixic acid and levofloxacin. The agar spot assay indicated an antimicrobial effect of the probiotic *L. reuteri* KUB-AC5 on all five-tested clinical strains demonstrated by the inhibition zone produced around the AC5 spot (Figure 1A). The CFS of AC5 anti-*Salmonella* activity was shown as a clear zone (Figure 1B). Mean and standard deviations of the inhibitory zone diameter are shown in Table 2.

The numbers of all STMCs were decreased when co-cultured with AC5 as observed in the co-culture growth assay. The inhibitory effect of AC5 on STMCs in liquid broth was observed at 8 h after inoculation (Figure 2). At 14 h after the inoculation, *Salmonella* numbers were decreased by approximately 10^3-fold in the co-culture assay compared to that of the mono-culture.

Table 1 Antibiotic resistance of five clinically isolated *S.* Typhimurium strains used in this study.

	Antibiotic resistance													
	S	TE	DO	KZ	CXM	CTX	CRO	CAZ	ATM	AMC	FEP	FOX	NA	LEV
STMC53	+	+	+	-	-	-	-	-	-	-	-	-	-	-
STMC78	+	+	-	+	+	+	+	+	+	+	-	+	-	-
STMC81	+	+	+	+	+	+	+	+	+	+	+	-	-	-
STMC101	+	+	+	+	+	+	+	+	+	+	+	-	-	-
STMC107	+	+	-	-	-	+	-	-	-	+	-	-	+	+

ATM, Aztreonam; AMC, Amoxicillin-clavulanic acid; CXM, Cefuroxime; CTX, Cefotaxime; CRO, Ceftriaxone; CAZ, Ceftazidime; DO, Doxycycline; FOX, Cefoxitin; FEP, Cefepime; KZ, Cephazolin; LEV, Levofloxacin; NA, Nalidixic acid; S, Streptomycin; TE, Tetracycline; +, resistance; -, susceptible

Table 2 Diameter of inhibitory zone created by *L. reuteri* KUB-AC5 against STMCs by agar spot and KUB-AC5 supernatant well diffusion assays.

Strain	Zone of inhibition (mm)[a]	
	Agar spot assay	Well diffusion assay
STMC 53	45.33 ± 0.58	10.67 ± 0.58
STMC 78	48.33 ± 0.58	10.67 ± 0.58
STMC 81	44.33 ± 1.15	9.67 ± 0.58
STMC 101	47.33 ± 2.52	10.00 ± 1.00
STMC 107	46.00 ± 1.00	11.33 ± 0.58
MRS (negative control)	0	6.00 ± 00

[a] Results are reported as mean ± standard deviation; STMC, clinically isolated *S.* Typhimurium; MRS, de Man, Rogosa, and Sharpe

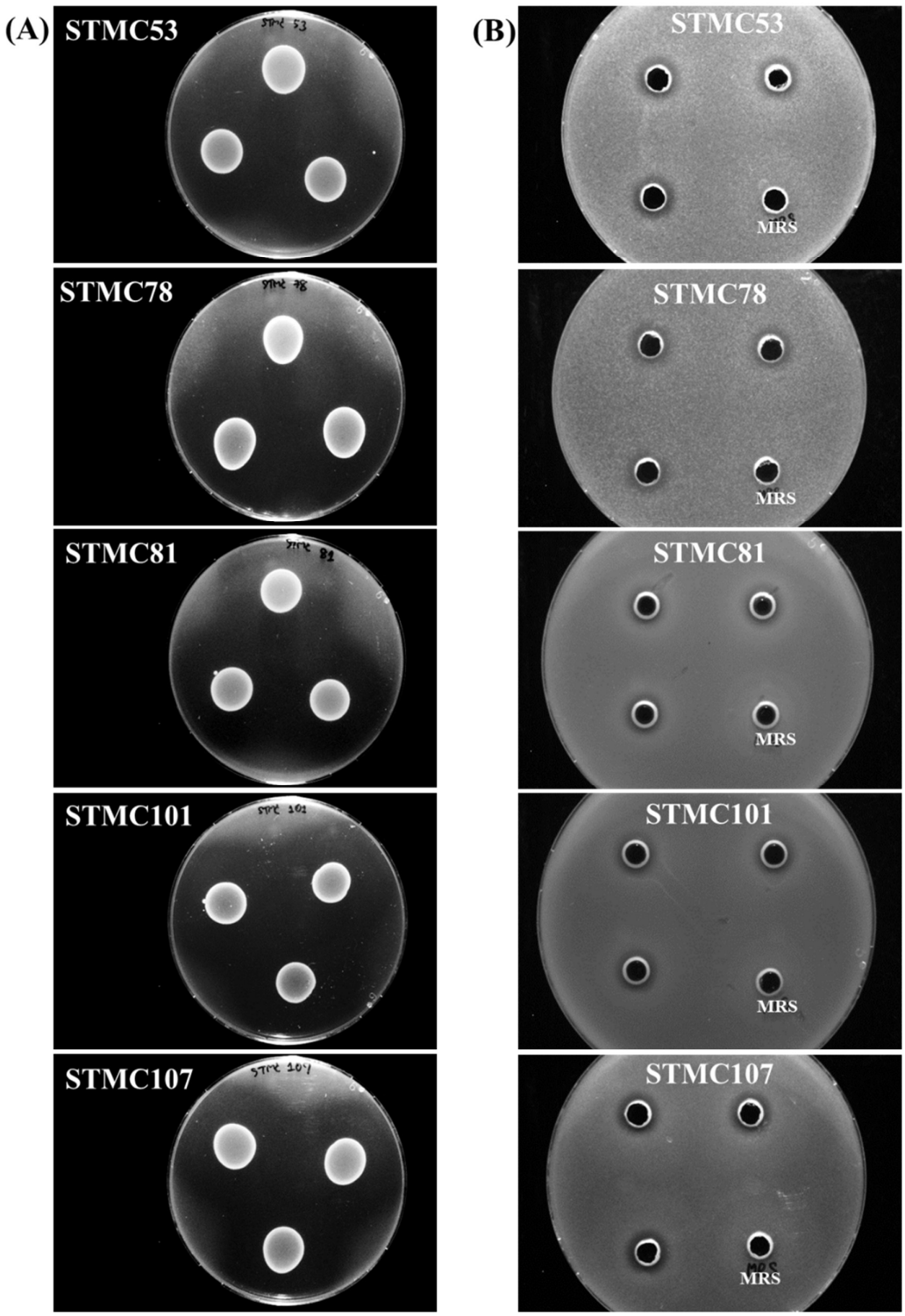

Figure 1 Antimicrobial effect of *L. reuteri* KUB-AC5 against *S.* Typhimurium strains: STMC53, STMC78, STMC81, STMC101, and STMC107. (A) Agar spot assay, (B) Cell-free supernatant agar well diffusion assay. All experiments were done in triplicate.

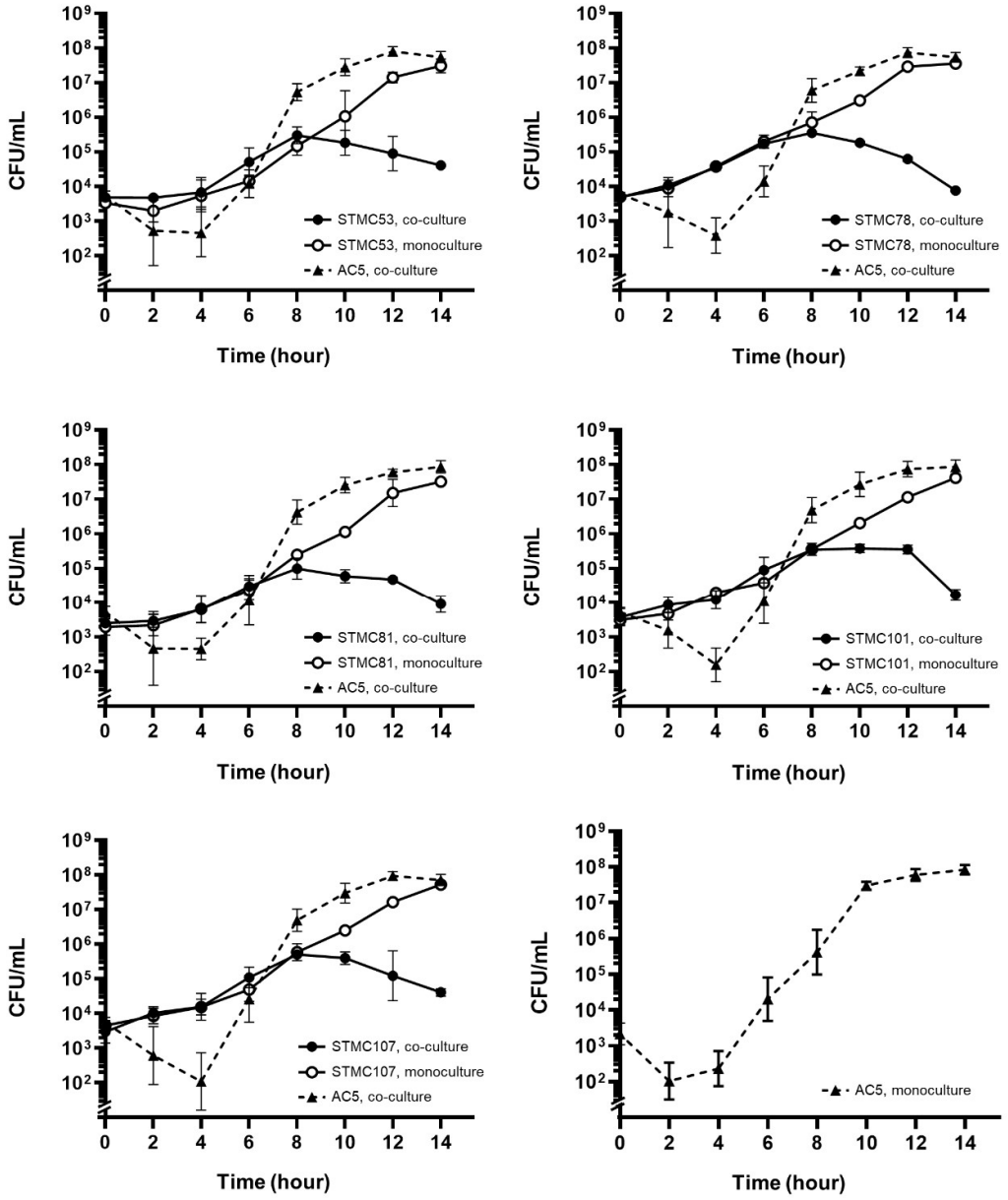

Figure 2 Growth of *L. reuteri* KUB-AC5 and STMC53, STMC78, STMC81, STMC101, and STMC107 in co-culture media (MH+MRS). The bacterial numbers are reported in CFU/mL. Data represent geometric means ± SDs of three independent experiments.

4. Discussion

Probiotic use is one of the most promising alternatives for prevention or treatment of NTS [30]. Adetoye showed anti-*salmonella* activity of *L. reuteri* isolated from cattle feces against *S. enterica* from cattle [31]. *L. reuteri* isolated *from* chickens exhibited antimicrobial activity against *S.* Enteritidis phagotype 4 and 28, *S*. Typhimurium 14028, and *S*. Pullorum 9120 by agar spot and well diffusion assay [32]. Nitisinprasert isolated *L. reuteri* KUB-AC5 from chicken intestine and demonstrated its probiotic proterties and antagonistic effect on several strains of *Salmonella* due to the production of the antimicrobial peptide KAC5 [23,33]. A recent report showed that AC5 can attenuate inflammatory conditions in both the gut and systemic site of *Salmonella*-infected mice [34]. AC5 consumption for 4 days after mice were infected with *S.* Typhimurium IR715 (nalidixic acid resistance derivative of ATCC 14028) significantly reduced *Salmonella* numbers in mouse intestine and spleen.

In this study, we found that all five clinical strains of *S.* Typhimurium (STMC53, 78, 81, 101 and 107) had the MDR phenotype. Our data showed that both viable cells and the cell-free supernatant of AC5 inhibits the growth of all STMCs (Figure 1A and 1B, respectively). Co-culture media containing MRS and MH broth were used to observe the growth inhibitory effect of AC5 on STMCs as previously described [35]. Our data demonstrated that AC5 could significantly inhibit the growth of all STMCs in a liquid medium. In the absence of AC5, all STMCs continued to grow beyond the 8 h time point and reached a stationary phase at 14 h after inoculation (Figure 2). This suggests that AC5 potentially creates an environment unsuitable for STMCs after 8 h in this co-culture setting. For example, a low pH environment (from the accumulation of lactic acid) or the appropriate concentration of antimicrobial substances generated by AC5. Different strains of *S.* Typhimurium especially with the clinically isolated strains could result in a different pathogenicity in their mammalian host [26]. Hence, more strains of STMC isolated from acute NTS patients in Thailand should be further investigated with regard to their susceptibility to AC5.

5. Conclusion

The probiotic *L. reuteri* KUB-AC5 exerts a strong inhibitory effect on the growth *in vitro* of five clinical STM strains collected from Thai NTS patients. Our data suggest that AC5 should be investigated more extensively with regard to its toxicity, dose, and duration of the administration for more in depth use in a further clinical trial.

6. Acknowledgements

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7. Conflicts of interest

The authors declare no conflict of interest.

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