

Full Length Research Paper

Visualization and colonization studies of a novel bacterium *Lactobacillus thermotolerans* on chicken cecum using fluorescence *in situ* hybridization-confocal laser scanning microscope (FISH-CLSM)

Abu Sadeque Md. Selim^{1*} and Atsushi Yokota²

¹Department of Animal Science and Nutrition, Faculty of Vet. Medicine and Animal Science, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur-1706, Bangladesh.

²Laboratory of Microbial Physiology, Research Faculty of Agriculture Hokkaido University Sapporo 060-8589, Japan.

Accepted 21 January, 2013

The direct visualization and colonization studies of *Lactobacillus thermotolerans* in cecal epithelium of chicken was attempted by applying fluorescence *in situ* hybridization in combination with confocal laser scanning microscope (FISH-CLSM) method. Chicken cecum (0.5 cm) as collected from White Leghorn were fixed, washed and then embedded in Tissue-Tek OCT compound. Vertical thin section (20 µm) of the fixed samples was prepared using Cryostat-3000, Leyca. Then the slices were fixed on gelatin-coated microscopic slides, air dried, and dehydrated in a series of increasing concentrations of ethanol followed by fluorescence *in situ* hybridization (FISH) and observed by confocal laser scanning microscope (CLSM). CLSM confirmed that bacteria were located in layers of the cecum tissue. Accordingly, the FISH technique demonstrated that the bacteria consist of cocci. The hybridized cells of target strain were found within the total bacteria on cecum surface, the results clearly demonstrated that *L. thermotolerans* are the inhabitants of chicken microbiota and colonized at a low number in terms of total bacteria.

Key words: *Lactobacillus thermotolerans*, FISH-CLSM method, chicken cecum.

INTRODUCTION

Lactobacillus thermotolerans is characterized as facultative anaerobic, Gram-positive, catalyze-negative, non-motile and non-spore-forming rods. The bacterium was isolated from chicken feces collected from Thai chicken (Niamsup et al., 2003). Later, the novel species was detected and monitored by Real Time PCR (Selim et al., 2005) and fluorescence *in situ* hybridization (Selim and Yokota, 2012) in real chicken feces. The bacterium may be a potential probiotic candidate. The use of probiotics to promote health and nutrition in animal production has been attracting a great deal of attention for a long time (Gilliand, 1990) and claims for improvement of growth, feed utilization, disease resistance and reduction of gut shedding of enteropathogenic bacteria (Ehrmann et al., 2002).

FISH has been successfully used in combination with confocal laser scanning microscopy (CLSM) and epifluorescence microscopy for the visualization of initial

and mature oral biofilm formed *in situ* (Al-Ahmad et al., 2007, 2009; Dige et al., 2007). Moreover, FISH is a recognized tool for the specific and sensitive identification of target organisms within complex microbial communities (Amman et al., 1995). Confocal Laser Scanning Microscope (CLSM) allows a three-dimensional noninvasive visualization of cells without distortion of their structure. Visualization of FISH labeled cells by CLSM has already been reported (Wimpenny et al., 2002). In combination with the nondestructive sectioning properties of CLSM, the FISH technique enables the visualization of the three-dimensional arrangement of the microbial population as well as the estimation of the quantitative

*Corresponding author. E-mail: anima_l2002@yahoo.com. Tel: 8801718370722; Fax: +88-02-9205333.

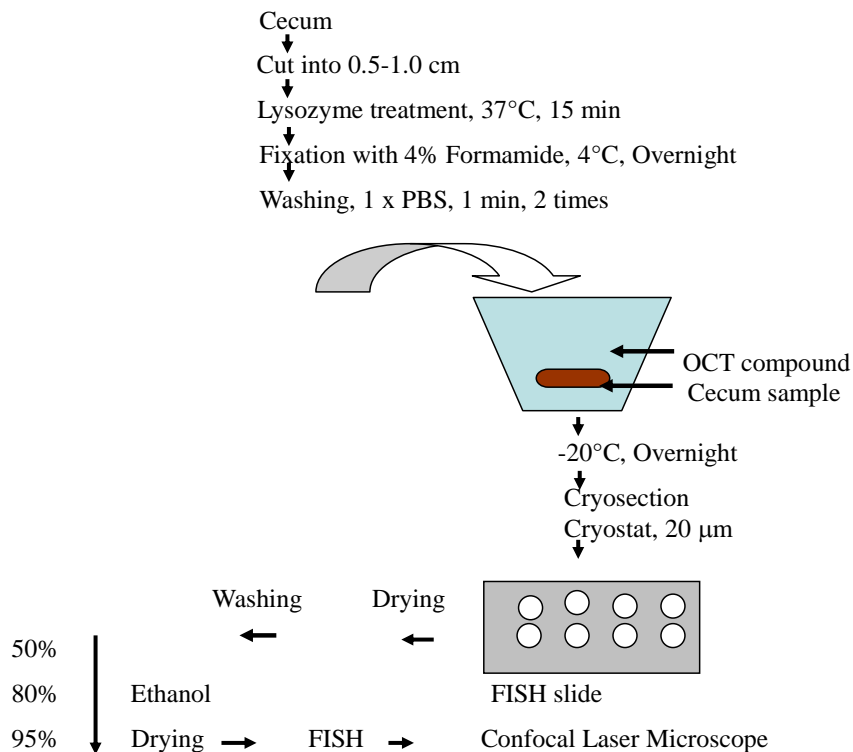


Figure 1. Flow chart of cecal sample preparation for confocal observation.

distribution of bacterium (Thurnheer et al., 2004).

Colonization of bacteria is one of the main factors for the microbial balance in the gut. Massive colonization of *Lactobacilli* in cecum was observed in a trial conducted by Fuller and Turvey (1971), whereas poor colonization was observed in another study conducted by Ehrmann et al. (2002). Overall, the tissue, cell, and molecular specificities of *Lactobacillus* adherence have remained poorly characterized, and the role of adherence in intestinal colonization by *Lactobacillus* has remained a controversial topic as reported by Tannock (1999).

Detection of bacterial 16S rRNAs, or the corresponding genes, in the gastrointestinal tracts of humans and different mammals by using PCR amplification or dot blot hybridization with specific probes has been successful for tracking pathogenic or probiotic strains and studying the gut ecology (Kaufmann et al., 1997; Kreader et al., 1995; Lin et al., 1997).

However, the visualization of bacteria using confocal microscope in combination with molecular technique is a new approach for the colonization study of bacterium in chicken intestine.

MATERIALS AND METHODS

Chickens and sampling

Chickens used in this study were the progeny of White

Leghorn (102 days old). They were obtained from Hokuren Federation of Agricultural Cooperatives, Hokkaido, Japan. The chickens were slaughtered and their cecal were collected by opening the chicken intestine using sterilized scissors. Then the cecum was kept in icebox and transferred to the laboratory for further processing.

Fixation and cryosectioning

The collected cecal (0.5 cm) were treated for 15 min at 37°C using lysozyme at the concentration of 1 mg/ml. Then the tissues were fixed by incubation in paraformaldehyde (4%) in phosphate buffered saline (pH 7.2) at 4°C for 16 h and subsequently washed twice in phosphate buffer. After fixation, the samples were embedded in Tissue-Tek OCT compound and frozen overnight at -20°C. Vertical thin section (20 µm) of the fixed samples was prepared using Cryostat-3000, Leyca. Then the slices were fixed on gelatin-coated microscopic slides, air dried, and dehydrated in a series of increasing concentrations of ethanol 50, 80 and 98%. The flow chart of the cecal sample preparation is shown in Figure 1.

FISH probe

The FISH probe for target bacteria was used according to the method of Selim and Yokota (2012). The probe

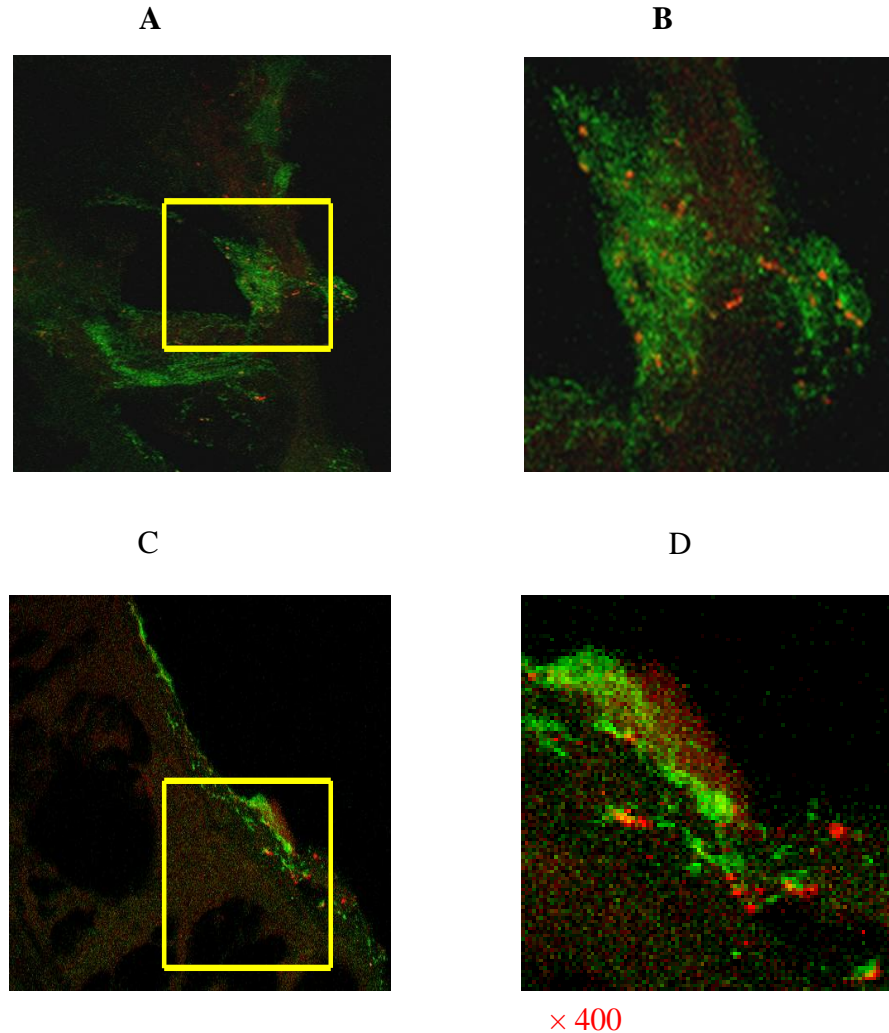


Figure 2. Confocal observation of total bacteria (green signal) and *L. thermotolerans* (red signal) in the fold of cecum surface (A, B) and fold of epithelium (C, D).

sequences were (L. ther) 5- 'CCGTCCGCCACTCGTTGGGA-3', labeled with Cy3 and FITC. Moreover, EUB 338 probe was used for total bacterium.

In situ hybridization

The cecal specimens were hybridized according to the method of Amann et al. (1990) and the specimen was examined with an LSM 410 confocal laser scanning microscope Carl Zeiss-410, equipped with an argon laser (a He Ne laser 543 nm, and a UV laser 488). Images were recorded by using simultaneous excitation of 488 and 543 nm lasers to distinguish the probe-stained cells. All image combination, processing, and analysis were performed with the standard software package provided by Zeiss. Processed images were printed out using the software package Adobe Photoshop, version 5.5.

RESULTS AND DISCUSSION

This is the first investigation of *L. thermotolerans* in the surface of chicken cecum where the FISH and CLSM techniques have been used, which turned out to be a powerful method for visualizing micro-organisms in their natural environment. The method was additionally improved by using CLSM, which allowed optical sectioning, three-dimensional reconstruction and therefore exact localization and observation of the spatial distribution of bacteria in the chicken cecum.

The confocal observations of *L. thermotolerans* in the epithelium of cecal surface and cecal fold are shown in Figure 2A, B, C and D respectively. In this study, *L. thermotolerans* (red color) was successfully visualized in the cecum using its newly designed probe (Selim and Yokota, 2012). The deep green cells indicate total bacteria visualized by EUB 338 probe. The photographs

(Figure 2A, B, C and D) suggest a low number of target strain present in the cecum, compared to those of total bacteria. The results also suggest that *L. thermotolerans* was colonized both on the cecal surface and in the cecum epithelium. In this study, *L. thermotolerans* was investigated in cecal surface where the FISH and CLSM techniques were used. Thus, the ecology of Lactobacilli in chicken gastro-intestinal tract can be understood much more effectively by the application of the FISH-CLSM method using various FISH probes for the detection of important species of Lactobacillus found in chicken gastro-intestinal tract.

The confocal observation strongly suggested that *L. thermotolerans* is a normal member of the chicken microbiota. This is the first successful visualization of the novel bacterium by the combined method of FISH and CLSM. It is quite interesting to visualize a bacterium directly in their original location. Figure 2A, B, C and D also showed that the number of *L. thermotolerans* is very low on the cecal surface and in the cecal fold as compared to total bacterium. This corresponds to the low percentage of *L. thermotolerans* that exist in feces and cecum sample as monitored by FISH (Selim and Yokota, 2012) and Real-Time PCR assay (Selim et al., 2005).

By using the EUB 338 probe, the total bacteria was also visualized, which gave confidence to the results obtained, especially when this study's target strain was observed. From Figure 2A, B, C and D, it seems that cells of *L. thermotolerans* are not localized in a particular position; they are spread on the surface. This study's result is in contrast with that of Ehrmann et al. (2002) who observed poor colonization of Lactobacilli. It is noted here that some strict anaerobic bacteria may not be detected by the FISH technique due to their low metabolic activity and the small number of rRNA copies, resulting in low signal intensity and false negative results (Pia et al., 2003).

In conclusion, direct visualization of bacteria with the FISH-CLSM technique provided additional support to the notion that *L. thermotolerans* is present in cecal surface with low number in terms of total bacteria. However, the FISH-CLSM method may be used for other similar researches of interest.

ACKNOWLEDGMENTS

The authors are grateful to Japanese Government (Monbukagakusho) Scholarship, Japan, for their financial assistance, and to Bangladesh Open University, Bangladesh, for the study leave granted to one of the authors. This research work was also supported by the Scientific Cooperation Program of the Japan Society for the Promotion of Science (JSPS).

REFERENCES

Al-Ahmad A, Follo M, Selzer AC, Hellwig E,

- Hannig, M. & Hannig, C. (2009a). Bacterial colonization of enamel in situ investigated using fluorescence in situ hybridization. *J Med Microbiol* 58, 1359–1366.
- Al-Ahmad, A., Wunder, A., Auschill, T. M., Follo, M., Braun, G., Hellwig, E. & Arweiler, N. B. (2007). The in vivo dynamics of *Streptococcus* spp., *Actinomyces naeslundii*, *Fusobacterium nucleatum* and *Veillonella* spp. in dental plaque biofilm as analysed by fivecolour multiplex fluorescence in situ hybridization. *J Med Microbiol* 56, 681–687.
- Amann R, Krumholz IL, Stahl DA (1990). Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J. Bacteriol.* 172: 762-770.
- Amann R, Ludwig IW, Schleifer KH (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* 59: 143–169.
- Dige, I., Nilsson, H., Kilian, M. & Nyvad, B. (2007). In situ identification of streptococci and other bacteria in initial dental biofilm by confocal laser scanning microscopy and fluorescence in situ hybridization. *Eur J Oral Sci* 115, 459–467.
- Ehrmann MA, Kurzak P, Bauer J, Vogel RF (2002). Characterization of lactobacilli towards their use as probiotic adjuncts in poultry. *J. Appl. Microbiol.* 92: 966-975.
- Fuller R. Turvey A (1971). Bacteria associated with the intestinal wall of the fowl (*Gallus domesticus*). *J. Appl. Bacteriol.* 34: 617-622.
- Gilliand SE (1990). Health and nutritional benefits from lactic acid bacteria. *FEMS Microbiol. Rev.* 87: 175-188.
- Kaufmann P, Pfefferkorn A, Teuber M, Meile L (1997). Identification and quantification of *Bifidobacterium* species isolated from food with genus-specific 16S rRNA-targeted probes by colony hybridization and PCR. *Appl. Environ. Microbiol.* 63: 1268-1273.
- Kreader CA, (1995). Design and evaluation of *Bacteroides* DNA probes for the specific detection of human fecal pollution. *Appl. Environ. Microbiol.* 61: 1171-1179.
- Lin C, Raskin L, Stahl DA (1997). Microbial community structure in gastrointestinal tracts of domestic animals: comparative analyses using rRNA-targeted oligonucleotide probes. *FEMS Microbiol. Ecol.* 22: 281-294.
- Niamsup P, Sujaya IN, Tanaka M, Sone T, Hanada S, Kamagata Y, Lumyong S, Assavanig A, Asano K, Tomita F, Yokota A (2003). *Lactobacillus thermotolerans* sp. nov., a novel thermotolerant species isolated from chicken faeces. *Int. J. Syst. Evol. Microbiol.* 53: 263-268.
- Pia S, Ingar O, Ulf G, Dirk T, Sascha W, Gilberto J. D, Leif T. Annette M (2003) Fluorescence *in situ* hybridization (FISH) for direct visualization of bacteria in periapical lesions of asymptomatic root-filled teeth. *Microbiol.* 149: 1095-1102.
- Selim ASM, Yokota A (2012). Monitoring of a novel

- bacterium, *Lactobacillus thermotolerans*, in chicken intestine, by fluorescence *in situ* hybridization (FISH). *Afric. J. Microbiol.* 11(69): 13436-1344.
- Selim, ASM, Piyanuch B, Teruo S, Apinya A, Wada M, Yokota A (2005). Development and assessment of a Real-Time PCR assay for rapid and sensitive detection of a novel bacterium, *Lactobacillus thermotolerans*, in chicken feces. *Appl. and Environ. Microbiol.* 71, 4214-4219.
- Tannock GW (1999). Analysis of the intestinal microflora: a renaissance. *Ant. van Leeuwenhoek.* 76: 265-278.
- Thurnheer T, Gmur R, Guggenheim B (2004). Multiplex FISH analysis of a six-species bacterial biofilm. *J. Microbiol. Meds.* 56: 37-47.
- Wimpenny J, Manz W, Szewzyk U (2000). Heterogeneity in biofilms. *FEMS Microbiol. Rev.* 24: 661- 671.