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# Applicability of a colorimetric method for evaluation of lactic acid bacteria with probiotic properties

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ABSTRACT

Rapid colorimetric methods using various indicator reagents have been developed to monitor bacterial viability. Here, we examined the applicability of a method based on the reduction of resazurin or water-soluble tetrazolium salt-8 (WST-8) to screen lactic acid bacteria (LAB) for growth, tolerance against bile acid and low pH. The resazurin reduction test proved unsuitable for screening LAB such as *Lactobacillus plantarum* and *Leuconostoc mesenteroides* since it reacted with acid present in the cultures. LAB growth could be indirectly quantified by measuring WST-8 reduction. This method proved more sensitive and quickly results than counting bacterial colony forming units and turbidity at 600 nm in the presence of bile and acid. Our results suggested that the WST-8-based method could be useful for the characterization of growth and tolerance of the lactic acid producing bacteria.



## 1. Introduction

The manufacture and marketing of fermented foods (dairy products and sausages) containing lactic acid bacteria (LAB) with probiotic properties have rapidly expanded worldwide (Leroy et al., 2006). LAB, including mainly lacotobacilli and bifidobacteria, are associated with a wide range of health-promoting properties such as increased intestinal function, neuroprotective effects, and anti-colitis and anti-obesity effects (Jang et al., 2014; Olivares et al., 2006; Ukibe et al., 2015). LAB need to be bile- and acid-tolerant since these probiotics are delivered into the gastrointestinal system that is highly acidic and contains high concentrations of bile and pancreatic enzymes (Chou and Weimer, 1999). The scientific validity of LAB as probiotics was first evaluated by characterizing acid and bile resistance in the intestinal tracts of human and animal hosts. Antibiotic resistance has become clinically important because of the potential for the transfer of antibiotic-resistance elements to other bacteria, including pathogenic bacteria (Klare et al., 2007). Thus, a rapid screening method for functional probiotic LAB is required for food-associated industries.

Colorimetric cell viability assays have been widely used as a rapid method for measuring the metabolic activities of both mammalian cells and microorganisms (Bernas and Dobrucki, 1999; Marshall et al., 1995). Microculture tetrazolium assays (MTAs) based on the reduction of tetrazolium salts to formazan by a mitochondrial dehydrogenase enzyme have been developed to measure the survival and proliferation of various bacteria and fungi (Tsukatani et al., 2014). Recently, the WST-8 [2-(2-methoxy-4-nitrophenyl-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] colorimetric method involving the formation of water-soluble formazan has become a very important tool in the study of bacterial cell viability and antimicrobial susceptibility. In this method, 2-methyl-1,4-naphthoquinone is reduced by bacteria to naphthohydroquinone, which then serves as an electron mediator for the reduction of WST-8 to formazan (optimum absorbance at 460 nm) (Hideyuki et al., 1999). The WST-8 method exhibits increased sensitivity and efficiency in detecting antimicrobial susceptibility compared with the standard broth microdilution methods recommended by the Clinical and Laboratory Standard Institute (CLSI) (Tsukatani et al., 2009). However, WST-8 has rarely been applied to LAB cell viability assays.

In this study, we describe the application of a colorimetric approach using the WST-8 method to screen for LAB possessing probiotic characteristics such as antimicrobial activity and acid and bile resistance.

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## 2. Materials and methods

### 2.1. Bacterial strains

*Lactobacillus plantarum* wikim18 (KFCC11588P) isolated from baechu (napa cabbage) kimchi was used as the typical LAB for identification of probiotic trait by the WST-8 method. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain wikim 18 is KT759680. *Escherichia coli* KCCM 11234 and *Leuconostoc mesenteroides* KCCM 11324 were obtained from the Korean Culture Center of Microorganisms (KCCM, Seoul, Korea). Strains were cultured overnight at 30 °C in de Man, Rogosa and Sharpe (MRS) and Luria-Bertani (LB) medium (Difco; Miller, Becton Dickinson, and Co., Sparks, MD, USA), respectively.

### 2.2. Resazurin assay

The concentration of viable cells was determined using the resazurin-based assay (Palomino et al., 2002) with the following modifications: 0.02% (wt/vol) resazurin sodium salt (Sigma Aldrich, USA) in distilled water was prepared and sterilized by filtration. *E. coli* KCCM 11234, *L. plantarum* wikim18, and *L. mesenteroides* KCCM 11324 were diluted $1/10^2$ to $1/10^5$ in each culture medium and then 200 μL of the culture dilutions was dispensed into the wells of sterile black 96-well plates (ThermoFisher Scientific, Waltham, MA, USA) containing 30 μL of the resazurin solution. Fluorescence was measured at 560 nm excitation and 590 nm emission using a microplate reader (Victor™ X2, PerkinElmer, Waltham, MA, USA) following 2, 4, 6, and 8 h incubation at 37 °C.

### 2.3. Characterization of L. plantarum wikim 18

#### 2.3.1. Comparison of WST-8 assay and cell count method

The WST-8 colorimetric method was performed with the cell counting kit-8 (CCK-8) including WST-8, 1-methoxy-PMS, and sodium chloride (Enzo Life Sciences, Farmingdale, NY, USA). *L. plantarum* wikim18 culture medium was adjusted to optical density (OD, at 600 nm) of 1.0 for this study. *L. plantarum* wikim18 diluted to and $1/10^1$–$1/10^5$ using MRS broth medium were used in comparative test of the cell counts, OD measurement and WST-8 assay. Two hundred microliters of diluted samples were dispensed to each well in sterile 96-well plates with 20 μL solution of CCK-8 or not. After 2h incubation at 30 °C, the absorbance of the samples in the presence and absence of the CCK-8 solution was measured at 450 and 600 nm, respectively, using a microplate reader (SPECTROstar Nano, BMG Labtech, Ortenberg, Germany). Also, *L. plantarum* wikim18 diluted to $1/10^2$ was incubated for 2, 4, 6, 8 and 21 h at 30 °C for a growth curve. Viable cell counts were determined by MRS agar plate method.

#### 2.3.2. Antibiotic activity by WST-8 assay

The dilutions (10–0.15 μg/ml) of erythromycin were prepared by two-fold serial dilution method and 100 μl of each dilution was placed into each well. *L. plantarum* wikim18 culture of OD 1.0 was diluted 100-fold and then 100 μL of the diluted cells mixed properly with 100 μL erythromycin of 96-well plates. *L. plantarum* wikim18 mixtures were incubated with 20 μL of CCK-8 solution under aerobic conditions at 30 °C for 21 h. The visible absorbance measurements were performed with a microplate reader at 450 nm.

#### 2.3.3. Survival in bile and acid

*L. plantarum* wikim18 was diluted to $1/10^2$ into MRS broth containing 0.3, 1 and 3% oxgall (Difco, USA) or bile salts (Sigma, USA) and adjusted to pH 2, 3, 4 and 7 using 0.1N HCl. Next, 200 μL of each *L. plantarum* wikim18 culture was incubated with 20 μL CCK-8 solution at 30 °C for 2, 4, 6, 8 and 21h.

### 2.4. Screening of LAB with probiotics properties

For the screening applications, LABs with the various species identified by 16S rRNA gene sequencing were isolated from homemade kimchi of different-region in Korea. Nineteen LABs containing *L. plantarum* wikim18 used as control of OD 1.0 were diluted to $1/10^2$ into MRS broth containing 0.3, 1 and 3% oxgall and pH 2, 3 and 4. Next, 200 μL of each strains culture was incubated for 2, 4, 6 and 8h at 30 °C with 20 μL CCK-8 solution.

### 2.5. Statistical analysis

Statistical evaluation was performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test as implemented in GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA). Differences were considered as statistically significant at $P < 0.05$.

## 3. Results and discussion

Colorimetric methods have been mainly used for testing the drug susceptibility of pathogenic bacteria such as *Mycobacterium tuberculosis* and *Staphylococcus* spp. (Martin et al., 2005). Resazurin assay that used often to assess bacterial or yeast in various environments was performed for determining LAB proliferation as the colorimetric assays in this study. The absorbance values of *E. coli* demonstrated a positive correlation with decreasing cell density and incubation time (Fig. 1A). In contrast, LAB strains did not show consistent results during the incubation period (Fig. 1B and C). The resazurin assay using resazurin-3H-phenoxazin-3-one 10-oxide (alamarblue) has been established as one of the best colorimetric methods for high-throughput quantification of microbial biofilms in a microtiter plate (Peeters et al., 2008); respiratory electron-transfer reactions occurring in viable cells reduce resazurin to resorufin resulting in the blue non-fluorescent dye changing to a fluorescent pink (Min and Kang, 2011). The resazurin assay results show that *E. coli* KCCM 11234 exhibited a pink color, indicating cell growth, however, the blue color changed to a yellowish brown following cultivation with the two LAB strains (data not shown). Resazurin color is also indicative of pH; the color is blue at pH > 6.8 and red at pH < 5.3; resorufin is red at pH > 6.8 and orange at pH < 5.3 (Min and Kang, 2011). These results indicate that resazurin is not suitable for the measurement of LAB cell proliferation because of the low pH caused by increased lactic acid.

*L. plantarum* species have been frequently reported as LAB with different potential probiotics characterization that exists in the food and environment (Zhang et al., 2014). In this study, *L. plantarum* wikim 18 was used for identification of probiotics traits. To investigate the applicability of this approach for assessing LAB cell proliferation, we compared the cell viability of *L. plantarum* wikim18 by measuring bacterial colony counts and absorbance in the presence and absence of the CCK-8 solution. The absorbance measurements of the $1/10^1$ to $1/10^5$ dilutions of *L. plantarum* wikim18 were approximately 0.2–1.1 ± 0.08 in the presence and 0.1–0.6 ± 0.15 in the absence of the CCK-8 solution. In addition, the log CFU/mL of these bacterial suspensions was 4.6–8.9 ± 0.09 (Fig. 2A). The diluted samples of *L. plantarum* wikim18 showed a higher increase in absorbance following 4–21 h incubation with or without the CCK-8 solution ($p > 0.05$; correlation analysis from XY column by GraphPad software). A wide range of absorbance at 450 nm was detected in cultures with WST-8 compared to 600 nm, although no significant differences were observed during 8h incubation of *L. plantarum* wikim18 diluted to $1/10^5$ in the presence