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Limosilactobacillus fermentum MG5091 and *Lactococcus lactis* MG4668 and MG5474 Suppress Muscle Atrophy by Regulating Apoptosis in C2C12 Cells

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Abstract: Muscular atrophy is a chronic muscle disease characterized by a loss of muscle mass and muscle weakness due to excessive protein breakdown relative to protein synthesis. Apoptosis is a major factor in sarcopenia and the final stage of muscle atrophy that occurs via various mechanisms. In this study, we evaluated the protective effects of cell-free supernatants (CFSs) from different lactic acid bacteria (LAB) strains in dexamethasone (DEX)-treated C2C12 cells, followed by probiotic properties. We found that *Limosilactobacillus fermentum* (*L. fermentum*) MG4263 and MG5091 and *Lactococcus lactis* (*Lc. lactis*) MG4668 and MG5474 inhibited muscle atrophy F-box (atrogin-1) and muscle-specific RING-finger protein-1 (MuRF-1) in DEX-treated C2C12 cells. In addition, LAB strains inhibited the expression of apoptotic proteins, such as Bcl-2-associated X (Bax)/Bcl-2 and caspase-3 in DEX-treated C2C12 cells. *L. fermentum* MG5091, *Lc. lactis* MG4668, and MG5474 showed high survival rates in gastrointestinal (GIT) conditions and high adhesion rate to HT-29 cells. The LAB strains were also assessed for hemolysis and toxicity in HT-29 cells to confirm their stability. The LAB strains showed no hemolytic activity and toxicity to HT-29 cells. Therefore, *L. fermentum* MG5091, *Lc. lactis* MG4668, and MG5474 suggest their potential as probiotics to be used as functional foods for the inhibition of muscular atrophy.

Keywords: lactic acid bacteria; dexamethasone; muscle atrophy; probiotics property

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1. Introduction

The elderly population is rapidly increasing worldwide due to aging [1]. Loss of skeletal muscle mass and muscle strength is the most prominent feature of aging in humans. Skeletal muscle is the largest tissue in the body and the most important protein reservoir [2]. Maintaining appropriate muscle protein levels is important to perform normal body functions [3]. Skeletal muscle mass is determined by the balance between muscle protein synthesis and breakdown [4]. Imbalance in protein metabolism leads to sarcopenia, which is a type of muscle atrophy characterized by a loss of skeletal muscle mass and function, physical disability, and reduced quality of life [5]. The World Health Organization designates sarcopenia as an official disease that is associated with excessive protein degradation due to aging and various chronic diseases, such as cancer, chronic obstructive pulmonary disease, and chronic heart disease [6,7]. Sarcopenia causes a loss in strength and muscle mass and increases the risk of physical disability and mortality in affected patients [8]. The incidence of sarcopenia is positively correlated with age, suggesting the need for medical and social services for the elderly; it further increases after the age of 50 years (from 5–13% at age 70 to 11–50% at age 80) [9]. Interestingly, sarcopenia exhibits a two-fold higher prevalence in females than in males, detrimentally affecting both the quality of life and lifespan of the affected individuals [10].

Lactic acid bacteria (LAB) are living microorganisms; in particular, some species, including *Lactobacillus* and *Lactococcus*, are used as probiotics and adhere to the intesti-

nal epithelial cells to remove harmful microorganisms and exert beneficial effects on the host [11,12]. The intricate interplay between the gut microbiota and host physiological homeostasis is crucial for the regulation of various essential processes, including the modulation of nutrient absorption, control of inflammatory responses, management of oxidative stress, regulation of immune function, and maintenance of anabolic balance in the body [13]. Although many reports have demonstrated the impact of LAB on intestinal health, recent studies have highlighted their potential in alleviating diabetes, obesity, heart disease, and liver disease [14]. Recent studies have also revealed the potential benefits of LAB in promoting gut microbiome balance and skeletal muscle growth [15]. Intestinal dysbiosis and consequent changes in the microbiome can lead to significant changes in skeletal muscle metabolism [16]. Consuming probiotics is one of the solutions that can restore gut microbiome balance and prevent gut microbiome imbalance [17]. *Lactiplantibacillus plantarum*, *Limosilactobacillus reuteri*, *Lactobacillus gasseri*, and *Lacticaseibacillus casei* have been reported to restore muscle-related parameters [18–20]. Although various LAB strains inhibit skeletal muscle atrophy, studies on some species are limited. Therefore, in this study, we aimed to evaluate the efficacies of various LAB strains in inhibiting dexamethasone (DEX)-induced skeletal muscle atrophy.

2. Materials and Methods

2.1. Preparation of Cell-Free Supernatants (CFSs) from Lactobacillus and Lactococcus Strains

Limosilactobacillus fermentum (*L. fermentum*) MG4263, MG5091, and MG5159 and *Lactococcus lactis* (*Lc. lactis*) MG4668, MG5049, MG5052, and MG5474 were obtained from MEDIOGEN (Jecheon, Republic of Korea). All LAB strains were cultured in the de Man, Rogosa and Sharp (MRS) broth (BD Bioscience, Franklin Lakes, NJ, USA) at 37 °C for 24 h. Then, the LAB strains were adjusted to an OD_{600} of 1.0 (10^8–10^9 CFU/mL) and sub-cultured at 37 °C for 18 h. CFS was obtained via centrifugation at 4000× *g* for 15 min at 4 °C and filtered using a 0.22 µm polytetrafluoroethylene membrane filter (ADVANTEC, Tokyo, Japan). All LAB strains were confirmed via *16S rRNA* gene sequencing (SolGent Co., Ltd., Daejeon, Republic of Korea) and registered on the National Center for Biotechnology Information database using the Basic Local Alignment Search Tool (Table 1).

Table 1. Accession numbers and origins of the LAB strains used in this study.

LAB	Strain	NCBI Accession Number	Origin
Limosilactobacillus fermentum	MG4263	OP102570.1	Human
	MG5091	OP102518.1	Food
	MG5159	MN435579.1	
Lactococcus lactis	MG4668	OP035505.1	Human
	MG5049	OP102495.1	Food
	MG5052	OP102497.1	
	MG5474	ON619520.1	

2.2. Cell Culture and Myotube Differentiation

C2C12 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in the Roswell Park Memorial Institute-1640 medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (P/S; Gibco) at 37 °C and 5% CO_2. C2C12 cells were cultured in a cell culture plate (1×10^5 cells/mL) for 24 h. To induce C2C12 cell differentiation into myotubes, the medium was replaced with a differentiation medium containing 2% horse serum and 1% P/S after five days. The differentiation medium was replaced every two days.

2.3. Cell Viability

Briefly, C2C12 cells were grown in a 96-well plate with the growth medium for 24 h, after which the medium was changed to a differentiation medium. After five days, cells were treated with 10% CFS of LAB and DEX (100 μM; Sigma-Aldrich, St. Louis, MO, USA) for 24 h. Cytotoxicity of the LAB strains was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [21]. MTT solution (0.25 mg/mL; Sigma-Aldrich) was added for 2 h. The formazan product was dissolved in dimethyl sulfoxide. Absorbance was measured at 550 nm using a microplate reader (BioTek, Winooski, VT, USA).

2.4. Western Blotting

C2C12 cells were seeded in a 12-well plate with the growth medium for 24 h, after which the medium was changed to differentiation medium. After five days, the cells were treated with CFS and exposed to DEX, as described in Section 2.3. Whole-cell lysates were prepared as previously described [22]. Cells were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and proteins were transferred to polyvinylidene fluoride membranes (0.45 μm; GenDEPOT, Katy, TX, USA). The membrane was washed thrice with Tris-buffered saline with 0.1% Tween-20 (TBS-T; GenDEPOT) and blocked with smart-block 5 min fast blocking buffer (Biomax, Guri-si, Gyeonggi-do, Korea). After blocking, the membrane was incubated with the muscle atrophy F-box (MAFbx/atrogin-1; Santa Cruz Biotechnology, Dallas, TX, USA), muscle-specific RING-finger protein-1 (MuRF-1; Santa Cruz Biotechnology), Bcl-2-associated X (Bax; Santa Cruz Biotechnology), Bcl-2 (Santa Cruz Biotechnology), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology), pro-caspase 3 (Cell Signaling Technology Beverly, MA, USA), and cleaved-caspase-3 (Cell Signaling Technology) primary antibodies diluted to 1:1000 overnight at 4 °C. After washing thrice with TBS-T, the membranes were incubated with secondary antibodies (1:5000 dilution; GenDEPOT) for 1 h at room temperature. Western blotting images were visualized using LuminoGraph III Lite (ATTO, Tokyo, Japan) with EzWestLumi plus mid-femto ECL solution (ATTO). Quantitative analysis was performed using ImageJ software (version 1.52a for Windows; NIH, Rockville, MD, USA).

2.5. Morphological Characterization of LAB Strains

Morphological characteristics of the LAB strains were determined using scanning electron microscopy (SEM; Hitachi S-4300SE, Tokyo, Japan), as previously described [23]. The cells were observed at 10,000× magnification.

2.6. Characterization of Carbohydrate Fermentation

To assess the availability of carbohydrates, the LAB strains were cultured on MRS agar (Difco, Sparks, MD, USA) for 18 h at 37 °C. Then, the carbohydrate fermentation profiles of the LAB strains were determined using the API 50 CHL Kit (BioMerieux, Craponne, France), according to the manufacturer's instructions. The extent of substrate hydrolysis was evaluated based on the intensity of color.

2.7. Hemolyltic Activity

Hemolytic activities of the LAB strains were assessed using Columbia agar (Oxoid, Basingstoke, UK) supplemented with 5% sheep blood (MB cell, Seoul, Republic of Korea). LAB strains were streaked onto agar plates and incubated at 37 °C for 48 h. Assessment of hemolytic activity involved the examination of red blood cell lysis in the vicinity of bacterial colonies on agar plates. Hemolysis was categorized into three types based on distinct visual characteristics: α-hemolysis: characterized by the presence of a greenish zone around the colonies, indicating partial lysis of red blood cells; β-hemolysis: characterized by a transparent zone, indicating the complete destruction of red blood cells; and γ-hemolysis: characterized by no noticeable zone, suggesting the complete absence of hemolysis [24].

2.8. Lactate Dehydrogenase (LDH) Release

Human colon adenocarcinoma (HT-29) cells (Korean Cell Line Bank, Seoul, Republic of Korea) were cultured in Dulbecco's modified Eagle's medium (Gibco) containing 10% FBS and 1% P/S at 37 °C in 5% CO_2. Cytotoxicity of the LAB strains was determined using the Quanti-LDH PLUS Cytotoxicity Assay Kit (Biomax, Seoul, Republic of Korea). HT-29 cells were cultured in a 96-well plate at a density of 2.5×10^4 cells/well. Subsequently, the LAB strains were treated with 10^6, 10^7, or 10^8 CFU/mL for 24 h. The culture medium (50 µL) was transferred to a fresh 96-well plate, and 50 µL of LDH substrate mix was added. After 30 min of incubation at 37 °C, the absorbance was measured at 450 nm using a microplate reader. LDH release was calculated using the following equation:

$$\text{LDH (\%)} = (\text{Sample} - \text{Low control})/(\text{High control} - \text{Background control}) \times 100 \quad (1)$$

Low control: cell culture supernatant of cells only; high control: cell culture supernatant of cells after lysis; background control: medium only.

2.9. Survival in the Gastrointestinal Tract (GIT)

Survival of LAB strains under conditions resembling the GIT environment was evaluated as previously described [25]. For simulated gastric fluid (SGF), LAB strains were treated with phosphate-buffered saline (PBS; pH 2.5) and 0.3% pepsin (Sigma-Aldrich) for 2 h at 37 °C. For simulated intestinal fluid (SIF), LAB strains were treated with PBS (pH 7.4) and 1% pancreatin-bile salt (Sigma-Aldrich) for 2.5 h at 37 °C. Following incubation, the viability of LAB strains was determined via live colony counts on the MRS agar plates.

2.10. Adhesion Assay on Intestinal Epithelial Cells

To evaluate the adhesion of the LAB strains to HT-29 cells, an adhesion assay was performed, as previously reported, with some modifications [26]. Briefly, HT-29 cells were seeded at 1.5×10^5 cells/well in a 12-well plate until a cellular monolayer was formed. Then, the cells were treated with LAB (1×10^8 CFU/mL) for 2 h, washed thrice, and lysed with 1 mL of PBS. The adhesion rate (%) was calculated by comparing the number of adherent cells to the initial number of viable cells using the following equation: log (adherent counts) CFU/mL/log (initial counts) CFU/mL × 100.

2.11. Statistical Analysis

All results are expressed as the mean ± standard error of three independent experiments. Statistical analysis was conducted using the Student's *t*-test with Prism 5.02 software (GraphPad Software, San Diego, CA, USA). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Cytotoxicity of the LAB Strains in DEX-Treated C2C12 Cells

DEX treatment significantly reduced the cell viability in a concentration-dependent manner (Figure 1A). After determining that 200 µM DEX drastically decreased the cell viability, subsequent experiments were performed using 100 µM DEX. As shown in Figure 1B, 10% CFS of all LAB strains exhibited no cytotoxicity (≥0.91-fold of the control). As shown in Figure 1C, *L. fermentum* MG4263, MG5091, and MG5159 and *Lc. lactis* MG4668, MG5049, MG5052, and MG5474 restored the cell viability (0.66–0.82-fold of the control) of DEX-treated C2C12 cells. Based on their cytotoxicity in DEX-treated C2C12 cells, four LAB strains (*L. fermentum* MG4263 and MG5091 and *Lc. lactis* MG4668 and MG5474) were selected to confirm muscle atrophy protein expression.

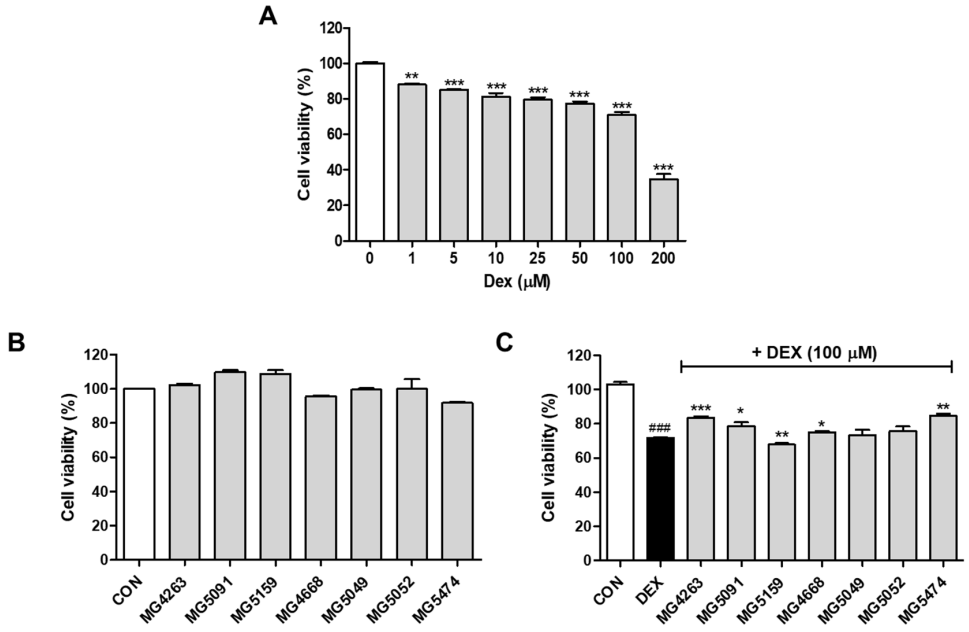

Figure 1. Cytotoxicity of the cell-free supernatants (CFSs) of different LAB strains with or without dexamethasone (DEX) treatment. C2C12 cells were treated with CFS of LAB strains (0–200 μM; (**A**)). Cell viability with 10% CFS without DEX (**B**) and with DEX (**C**). Data were analyzed using the Student's *t*-test. ### $p < 0.001$ vs. CON and * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.01$ vs. DEX treatment.

3.2. Inhibitory Effects of the LAB Strains on Muscle Atrophy Protein Expression Levels in DEX-Treated C2C12 Cells

Western blotting was performed to confirm the effects of CFSs from the selected LAB strains on muscle atrophy protein expression. Protein expression levels of MuRF-1 and atrogin-1 were significantly increased in DEX-treated C2C12 cells compared to the control cells (Figure 2). Expression levels of MuRF-1 were significantly decreased by all LAB strains (0.39–0.57-fold of DEX treatment; Figure 2A). In addition, the expression levels of atrogin-1 were significantly decreased by all LAB strains (0.47–0.80-fold of DEX treatment, $p < 0.01$), except *L. fermentum* MG4263 (Figure 2B).

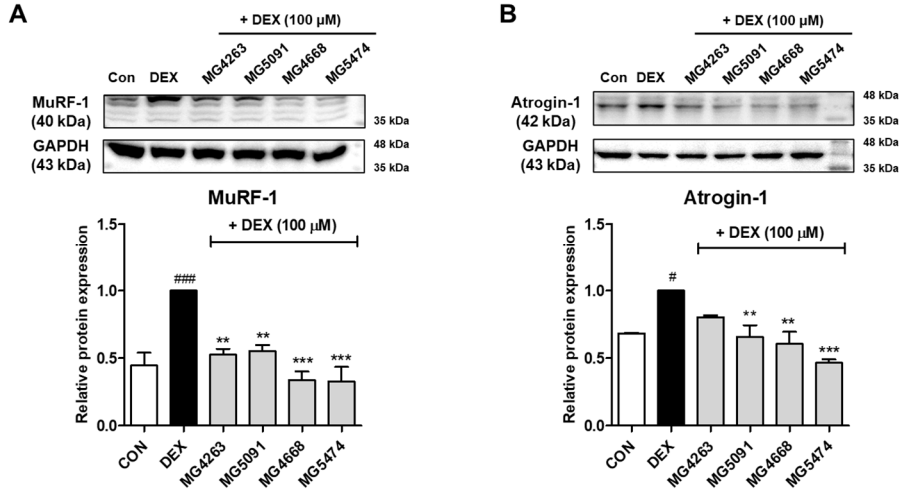

Figure 2. LAB strains inhibit muscle-specific RING-finger protein-1 (MuRF-1) (**A**) and muscle atrophy F-box (MAFbx/atrogin-1) (**B**) protein expression in DEX-treated C2C12 cells. Protein expression is normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data were analyzed using the Student's *t*-test. # $p < 0.05$ and ### $p < 0.001$ vs. CON and ** $p < 0.01$ and *** $p < 0.01$ vs. DEX treatment.

3.3. Inhibitory Effects of the LAB Strains on Apoptotic Protein Expression Levels in DEX-Treated C2C12 Cells

To assess the inhibitory effects of CFS on apoptotic protein expression, the expression levels of Bax/Bcl-2 and caspase-3 were determined in DEX-treated C2C12 cells (Figure 3). DEX treatment significantly upregulated the expression levels of Bax/Bcl-2 and cleaved-caspase-3. Expression levels of Bax/Bcl-2 were reduced by LAB strains (0.43–0.83-fold of DEX treatment), except *L. fermentum* MG4263 (Figure 3A). In addition, the expression levels of cleaved-caspase-3 were significantly decreased by all LAB strains (0.38–0.67-fold of DEX treatment; $p < 0.05$; Figure 3B).

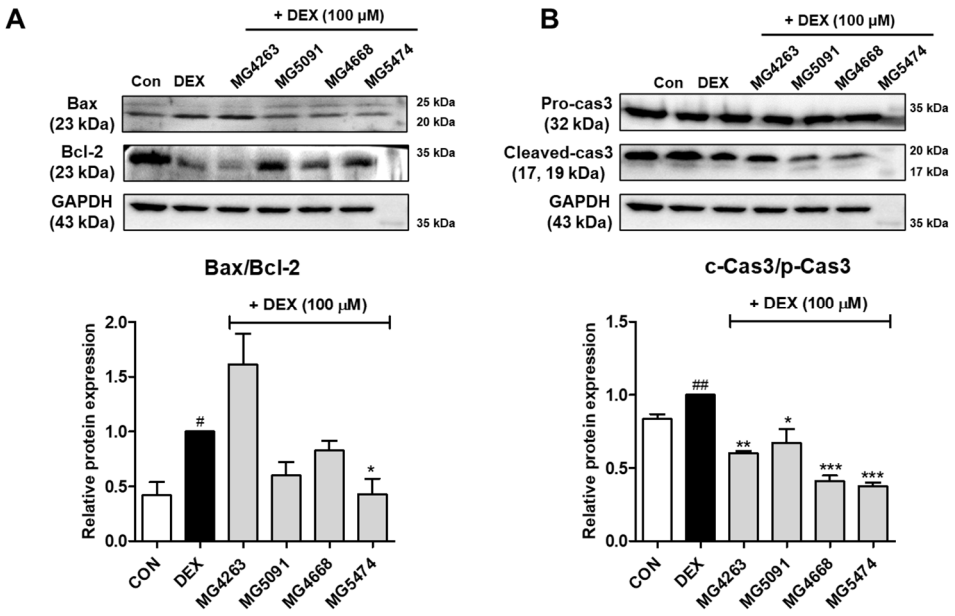

Figure 3. LAB strains inhibit Bax/Bcl-2 (**A**) and caspase-3 (**B**) expression in DEX-treated C2C12 cells. Protein expression is normalized to that of GAPDH. Data were analyzed using the Student's *t*-test. # $p < 0.05$ and ## $p < 0.01$ vs. CON and * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. DEX treatment.

3.4. Identification of the LAB Strains

The selected LAB strains were further assessed using SEM, which confirmed that all strains were in the form of bacilli (*L. fermentum* MG5091) and cocci (*Lc. lactis* MG4668 and MG5474, Figure 4). Evaluation of the carbohydrate fermentation profiles of the LAB strains using the API web software revealed that they all used L-arabinose, D-ribose, D-galactose, D-glucose, D-fructose, esculin, D-maltose, D-melibiose, D-sucrose, D-trehalose, and D-raffinose (Table 2). Each strain had a different ability to use residual carbohydrates, and the strains were identified based on *16S rRNA* gene sequencing results.

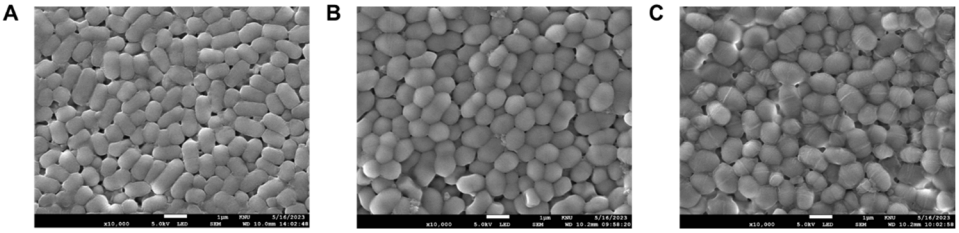

Figure 4. Scanning electron microscopy (SEM) micrographs of the *Limosilactobacillus fermentum* MG5091, (**A**) *Lactococcus lactis* MG4668 (**B**), and *Lc. lactis* MG5474 (**C**) at 10,000× magnification.

Table 2. Carbohydrate fermentation profiles of the LAB strains.

Carbohydrates	MG5091	MG4668	MG5474
L-Arabinose	+	+	+
D-Ribose	+	+	+
D-Xylose	-	+	+
D-Galactose	+	+	+
D-Glucose	+	+	+
D-Fructose	+	+	+
D-Mannose	-	+	+
D-Mannitol	-	+	-
N-Acetylglucosamine	-	+	+
Amygdalin	-	+	-
Arbutin	-	+	+
Esculin	+	+	+
Salicin	-	+	+
D-Cellobiose	-	+	+
D-Maltose	+	+	+
D-Lactose	-	+	+
D-Melibiose	+	+	+
D-Sucrose	+	+	+
D-Trehalose	+	+	+
D-Raffinose	+	+	+
Starch	-	+	+
Gentiobiose	-	+	+
Gluconate	+	+	+
5-keto-gluconate	+	-	-

- indicates that the strain could not grow; + indicates strain growth. All strains were negative for glycerol, erythritol, D-arabinose, L-xylose, D-adonitol, methyl-β-D-xyloside, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl-α-D-mannoside, methyl-α-D-glucoside, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, and 2-keto-gluconate.

3.5. Cytotoxicity of the LAB Strains in HT-29 Cells

To determine the safety of *L. fermentum* MG5091 and *Lc. lactis* MG4668 and MG5474, which inhibited muscle atrophy protein expression in C2C12 cells, their cytotoxicity was assessed in HT-29 cells. *L. fermentum* MG5091 and *Lc. lactis* MG4668 and MG5474 exhibited no cytotoxicity (0.95–1.15-fold of control) in HT-29 cells up to 1×10^8 CFU/mL (Figure 5).

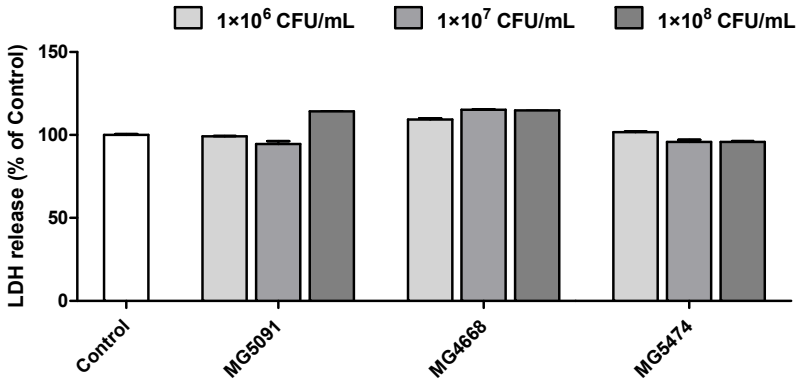

Figure 5. Cytotoxicity of the LAB strains in HT-29 cells. HT-29 cells were treated with LAB strains (10^6–10^8 CFU/mL). Cytotoxicity was subsequently confirmed via LDH release.

3.6. Viability in Simulated Gastrointestinal Fluid and Adhesion of LAB Strains to HT-29 Cells

To confirm the maintenance of viability during gastrointestinal transit, viability of the selected strains was assessed by culturing them in SGF and SIF (Table 3). The viability of the strains in SGF ranged from 36.6 to 98.2%. *L. fermentum* MG5091 and *Lc. lactis* MG4668 and MG5474 exhibited viabilities ranging from 37.5 to 96.8% in SIF. *L. fermentum* MG5091 showed higher survival rates than *Lc. lactis* MG4668 and MG5474. Next, we confirmed the

ability of these cells to adhere to intestinal epithelial cells. *L. fermentum* MG5091 and *Lc. lactis* MG4668 and MG5474 adhered to HT-29 cells at 74.0–89.7% compared to the initial count (Table 3).

Table 3. Survival rates of LAB strains in simulated gastrointestinal fluid and their adhesion to HT-29 cells.

Experiments		*L. fermentum*	*Lc. lactis*	
		MG5091	**MG4668**	**MG5474**
GIT (Log CFU/mL)	Initial	8.14 ± 0.02	7.59 ± 0.03	7.52 ± 0.01
	SGF	7.99 ± 0.03	2.78 ± 0.01	7.18 ± 0.02
	SIF	7.88 ± 0.01	2.85 ± 0.00	7.05 ± 0.00
Reference		- [1]	[25]	[25]
Adhesion (Log CFU/mL)	Initial	9.19 ± 0.02	8.66 ± 0.07	8.41 ± 0.02
	Adherent	6.99 ± 0.10	6.41 ± 0.03	7.55 ± 0.07
Adhesion rate (%)		76.0 ± 1.12	74.0 ± 0.33	89.7 ± 0.77
Reference		-	-	[12]

[1] All data were from the present study. GIT, gastrointestinal tract; SGF, simulated gastric fluid; SIF, stimulated intestinal fluid.

4. Discussion

With the global population transitioning to a super-aged society, the interest in geriatric diseases, including sarcopenia, hypertension, and degenerative neurological disorders, is rapidly increasing. Muscular atrophy is a chronic progressive muscle disease that reduces the quality of life of affected patients and is characterized by a loss of muscle mass, muscle weakness, and disability due to excessive protein breakdown relative to protein synthesis [13]. Therefore, maintenance of muscle health, the principal reservoir of proteins within the body, promotes healthy aging, ensures sufficient energy supply, and alleviates the risk of metabolic disorders [2,27]. Understanding and managing this condition are crucial for improving the quality of life of individuals with muscular atrophy worldwide. Currently, only non-pharmacological therapies (exercise, physical therapy, and diet) are used concurrently for treatment, as there are no US Food and Drug Administration-approved drugs for treating sarcopenia [28]. Due to the lack of effective medications, it is essential to identify safe materials that can maintain skeletal muscle mass by controlling muscle degradation without any side effects. Probiotic supplementation is effective to change the proportion and distribution of the gut microbiome. *L. fermentum* and *Lc. lactis*, generally recognized as safe (GRAS)-status microorganisms, are known to be safe when ingested up to concentrations of 10^9–10^{11} CFU/day [29]. Recent studies have reported that gut probiotics can influence skeletal muscle metabolism through the formation of ATP through metabolic activity and may play a key role in maintaining energy production during exercise [20]. Also, ingestion of LAB improves muscle mass and exercise capacity in healthy people and reduces muscle loss in cancer patients [30]. Previous studies have demonstrated the beneficial effects of the oral administration of *Lactobacillus* strains on muscle-related parameters in animal models. For instance, oral administration of *L. plantarum* TWK10 increases the muscle mass and improves the exercise capacity of the Institute of Cancer Research mice [20]. Similarly, *L. reuteri* 100-23 and *L. gasseri* 311,476 significantly reduce the expression levels of muscle atrophy markers, such as MuRF-1 and atrogin-1 [19]. *L. casei* LC122 enhances muscle strength in C57BL/6 mice [18]. These studies highlight the therapeutic potential of *Lactobacillus* species in alleviating muscle atrophy by promoting muscle mass gain.

Muscle atrophy primarily occurs due to multiple factors, including muscle cell damage induced by elevated levels of oxidative substances, reduced production and regeneration of muscle proteins due to decreased expression of stress proteins, and promotion of muscle protein degradation via the activation of the ubiquitin–proteasome pathway [31]. The

ubiquitin–proteasome pathway is primarily involved in skeletal muscle protein degradation [32]. MuRF-1 and atrogin-1 are skeletal muscle-specific ubiquitin ligases, whose activation induces the loss of muscle mass, that are used as markers of muscle atrophy [33]. DEX, a potent anti-inflammatory and immunosuppressive agent, is used for its biological effects. However, high doses and prolonged use of DEX impair protein synthesis and induce muscle mass loss [34]. DEX has been extensively used in both in vitro and in vivo models to elucidate the fundamental mechanisms underlying muscle atrophy [35]. In this study, DEX increased, whereas *L. fermentum* MG5091, *Lc. lactis* MG4668, and MG5474 significantly decreased the expression levels of MuRF-1 and atrogin-1.

Skeletal muscle apoptosis is a major indicator of sarcopenia that inhibits cell proliferation and induces apoptosis via various mechanisms [36]. Apoptosis is the final stage of muscle loss and atrophy [9]. During early apoptosis, Bax activation leads to an increase in the Bax/Bcl-2 ratio, resulting in the initiation of the mitochondrial apoptotic signaling pathway and activation of the final effector caspases [37]. Caspases, also known as cysteine-aspartic proteases, are essential enzymes involved in the regulation of apoptosis and skeletal muscle wasting under diverse conditions [9]. Caspases in the nucleus and mitochondrial membranes of cells play important roles in determining the morphological characteristics of apoptosis [38]. Activated caspase-3 induces DNA cleavage by stimulating apoptosis in cells [39]. In this study, *L. fermentum* MG5091, *Lc. lactis* MG4668, and MG5474 inhibited the expression of the pro-apoptotic protein, Bax, and the activation of the apoptotic protein, caspase-3, in DEX-treated C2C12 cells. In particular, the levels of these apoptotic factors were significantly decreased by *Lc. lactis* MG5474 compared to that by *L. fermentum* MG5091 and *Lc. lactis* MG4668. These results suggest that LAB strains suppress sarcopenia by inhibiting muscle atrophy and apoptotic protein expression in C2C12 cells.

Probiotics have beneficial properties, such as tolerance to gastrointestinal conditions, adherence to intestinal epithelial cells, and safety [12]. Hemolysis is a major virulence factor for pathogenic bacteria and a vital safety property of probiotics [40]. We proved the safety of *Lc. lactis* MG5474 in our previous study [12]. Here, *L. fermentum* MG5091 and *Lc. lactis* MG4668 did not exhibit any hemolytic activity (Figure S1) and were not cytotoxic to HT-29 cells. Thus, *L. fermentum* MG5091, *Lc. lactis* MG4668, and MG5474 were confirmed to be safe for practical use. When consumed, probiotics must survive in the human stomach and gastrointestinal tract and adhere to the colon to provide health benefits to the host [41]. *L. fermentum* MG5091 and *Lc. lactis* MG5474 cells exhibited excellent adhesion to HT-29 cells in both SGF and SIF. In contrast, the survival rate of *Lc. lactis* MG4668 was lower than that of *L. fermentum* MG5091 and *Lc. lactis* MG5474. In our previous study, cryoprotectants, such as sodium alginate and pumpkin powder, improved the survival rate of strains in SGF and SIF [42]. Therefore, probiotic protectants may aid in increasing the survival rate of *Lc. lactis* MG4668 in GIT; however, this requires validation in future studies.

In this study, *L. fermentum* MG5091, *Lc. lactis* MG4668, and MG5474 were evaluated for their safety, including adhesion to intestinal epithelial cells and hemolytic activity. Our results indicate that these strains are safe for use as probiotics. Moreover, we confirmed the effects of *L. fermentum* MG5091, *Lc. lactis* MG4668, and MG5474 strains on muscle atrophy and apoptotic protein expression in C2C12 cells. However, the specific mechanism of sarcopenia remains unknown and requires further investigation in future studies. Moreover, animal studies and clinical trials are necessary to verify the inhibitory effects of these probiotic strains on muscle atrophy.

5. Conclusions

In summary, LAB strains suppressed muscle atrophy and apoptotic proteins in DEX-treated C2C12 cells. *L. fermentum* MG5091 and MG4668 and *Lc. lactis* MG5474 showed high viability in GIT conditions and high adhesion to HT-29 cells. These strains are also safe to be used as probiotics, with no hemolytic activity. Our results suggest *L. fermentum* MG5091 and MG4668 and *Lc. lactis* MG5474 as potential probiotics for the prevention of sarcopenia.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9070659/s1, Figure S1: Hemolysis activity of LAB strains.

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