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Running Title (within 10 words)	LAB-bioconverted whey exhibits anti-adipogenic effect
Author	Ji Soo Lee†, In Kyung Hyun†, Ji-Won Yoon, Hye-Jin Seo, Seok-Seong Kang
Affiliation	Department of Food Science and Biotechnology, College of Life Science and Biotechnology, Dongguk University-Seoul, 10326, Korea
Special remarks – if authors have additional information to inform the editorial office	†These authors contributed equally to this work
ORCID (All authors must have ORCID) **https://orcid.org**	Ji Soo Lee (https://orcid.org/0000-0002-4579-6570) In Kyung Hyun (https://orcid.org/0000-0002-9417-6757) Ji-Won Yoon (http://orcid.org/0000-0002-5554-2259) Hye-Jin Seo (https://orcid.org/0000-0002-0460-6857) Seok-Seong Kang (https://orcid.org/0000-0001-7029-9122)
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Author contributions (This field may be published.)	Conceptualization: Kang SS. Data curation: Lee JS, Hyun IK, Yoon JW, Seo HJ, Kang SS. Formal analysis: Lee JS, Hyun IK, Yoon JW, Seo HJ. Methodology: Yoon JW, Seo HJ, Kang SS. Software: Lee JS, Hyun IK. Validation: Lee JS, Hyun IK, Yoon JW, Seo HJ. Investigation: Lee JS, Hyun IK, Yoon JW, Seo HJ.

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6 **CORRESPONDING AUTHOR CONTACT INFORMATION**

For the <u>corresponding</u> author (responsible for correspondence, proofreading, and reprints)	**Fill in information in each box below**
First name, middle initial, last name	Seok-Seong Kang
Email address – this is where your proofs will be sent	sskang@dongguk.edu
Secondary Email address	Kangelo72@gmail.com
Postal address	Department of Food Science and Biotechnology, College of Life Science and Biotechnology, Dongguk University-Seoul, 32 Dongguk-ro, Ilsandong-gu, Goyang-si, Gyeonggi-do, 10326, Korea
Cell phone number	+82-10-3669-6409
Office phone number	+82-31-961-5150
Fax number	+82-31-961-5108

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Abstract

Microbial bioconversion using lactic acid bacteria provides several human health benefits. Although whey and whey-derived bioactive compounds can contribute to an improvement in human health, the potential anti-obesity effect of whey bioconversion by lactic acid bacteria has not been well studied. This study aimed to investigate whether bioconversion of whey by *Pediococcus pentosaceus* KI31and *Lactobacillus sakei* KI36 (KI31-W and KI36-W, respectively) inhibits 3T3-L1 preadipocyte differentiation. Both KI31-W and KI36-W reduced intracellular lipid accumulation significantly, without decreasing 3T3-L1 preadipocyte proliferation. In addition, obesity-related transcription factor (peroxisome proliferator-activated receptor γ) and genes (adipocyte fatty acid-binding protein and lipoprotein lipase) were down-regulated significantly in 3T3-L1 cells in the presence of KI31-W and KI36-W. Collectively, these results suggest that bioconversion of whey by lactic acid bacteria exhibits anti-adipogenic activity and may be applied as a therapeutic agent for obesity.

Keywords: bioconversion, whey, lactic acid bacteria, obesity, 3T3-L1 preadipocyte

Introduction

Whey is the principal by-product of cheese manufacture. The huge quantities of whey produced by the dairy industry during cheese-making are difficult to dispose of, creating a serious environmental issue (Domingues et al., 1999; Marshall, 2004). However, whey contains a rich and heterogeneous mix of proteins, including β-lactoglobulin, α-lactalbumin, bovine serum albumin, glycomacropeptide, immunoglobulins, and lactoferrin (Walzem et al., 2002). Thus, this protein provides antimicrobial activity, immunomodulation, improved muscle strength, and lessens several risk factors for metabolic diseases (Bjornshave and Hermansen, 2014; Marshall, 2004).

Bioconversion is known as a structural modification of chemical compounds (Asha and Vidyavathi, 2009). The recognition of bioconversion by microbes as an important tool has increased in the chemical, pharmaceutical, cosmetic, and food industries (Hegazy et al., 2015; Smitha et al., 2017). Lactic acid bacteria (LAB) have been traditionally used as the starter culture in fruits, vegetables, and dairy products, as well as probiotics (Sornplang and Piyadeatsoontorn, 2016). In addition, various enzymes, such as β-glucosidase, produced by LAB also utilize nutritional substances, providing benefits to the host (Lin et al., 2002). Furthermore, LAB associated with the bioconversion provide health-promoting attributes (Boguta et al., 2014; Lee and Paik, 2017).

Obesity is defined as the excessive accumulation of fat in the adipose tissue as a result of the imbalance between energy intake and expenditure (Spiegelman and Flier, 2001). The surplus energy derived from the metabolism of dietary carbohydrates, fats, and proteins leads to hypertrophic and hyperplasic adipose tissue expansion (Yang et al., 2019). The dramatic increasing incidence of metabolic diseases, including obesity, imposes a growing economic and medical burden on societies. Therefore, the biological components produced during whey

bioconversion by LAB might be beneficial for metabolic diseases, such as obesity. In this study, we investigated whether bioconversion of whey using two LAB, *Pediococcus pentosaceus* and *Lactobacillus sakei*, reduces adipogenesis of preadipocytes.

Materials and Methods

Bioconversion of whey by LAB

Two LAB isolated from kimchi (*P. pentosaceus* KI 31 and *L. sakei* KI 36) were kindly provided by Dr. Sang-Dong Lim (Korea Food Research Institute, Wanju, Korea). Both LAB were cultured in de Man–Rogosa–Sharpe (MRS) medium (Neogen, Lansing, MI, USA) at 37°C. Whey bioconversion medium was prepared by adding 10% (w/v) whey powder (Samik Dairy & Food Co., Ltd., Seoul, Korea) to distilled water, followed by pasteurization at 80°C for 1 min. The whey bioconversion medium was inoculated by either *P. pentosaceus* KI 31 or *L. sakei* KI 36 (1%) and incubated at 37°C for 24 h. After incubation, cell-free supernatants were collected by centrifugation and adjusted to pH 6.5 with 1N NaOH. The filtrate was obtained by passing the cell-free supernatants through a syringe filter. Protein concentrations of *P. pentosaceus* KI 31- and *L. sakei* KI 36-bioconverted whey (KI 31-W and KI 36-W, respectively) were measured using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA).

Cell culture and differentiation of preadipocytes

The mouse preadipocyte cell line, 3T3-L1, was purchased from the Korean Cell Line Bank (Seoul, Korea) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (BCS), 100 U/mL penicillin, and 100 μg/mL streptomycin (HyClone, Logan, UT, USA) at 37°C in humidified air containing 5% CO_2. For adipocyte differentiation, 2-day post-confluent 3T3L-1 cells were stimulated with different concentrations

of KI 31-W or KI 36-W in DMEM supplemented with 10% BCS, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 5 μg/mL insulin (day 0). The cells were re-stimulated with KI 31-W or KI 36-W on day 3 and re-fed every 3 days with DMEM supplemented with 10% BCS and 5 μg/mL insulin. After incubation for 6 days, the medium was replaced with DMEM supplemented with 10% fetal bovine serum and antibiotics, and the cells were further incubated for 3 days.

Cell viability assay

The 3T3L-1 cells were seeded on a 96-well culture plate and grown for 48 h. After the treatment with the different concentrations of KI 31-W or KI 36-W for 24 h, MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) solution (0.5 mg/mL) was added to the medium and incubated at 37°C for 4 h. The resulting formazan dark purple crystal products were dissolved in 100 μL of dimethyl sulfoxide and the absorbance was measured at 570 nm using a microplate reader (Allsheng, Hangzhou, China).

Oil Red O staining

3T3-L1 cells treated with KI 31-W or KI 36-W were washed with phosphate-buffered saline and fixed with 10% formaldehyde for 1 h. After removing the formaldehyde, the cells were washed with 60% isopropanol and stained with Oil Red O solution (Sigma–Aldrich, St. Louis, MO, USA) for 30 min. Quantification of lipid accumulation was measured at 492 nm using a microplate reader (Allsheng). Microscopic images were also captured to visualize red oil droplet staining in differentiated 3T3-L1 cells treated with or without KI 31-W or KI 36-W. In addition, the lipid accumulation of 3T3-L1 cells treated by non-bioconverted whey was also determined by Oil Red O staining described above.

SDS-PAGE

Non-bioconverted whey and bioconversion of whey by *P. pentosaceus* KI 31 or *L. sakei* KI 36 (15 μL each) were loaded on the gel and separated using 10% SDS-PAGE. The separated proteins were visualized by Coomassie blue staining.

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

After the differentiation of 3T3L-1 cells treated with the different concentrations of KI 31-W or KI 36-W, total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, complementary DNA was synthesized using total RNA (5 μg), random hexamers, and reverse transcriptase (Promega, Madison, WI, USA). Afterward, real-time PCR was performed with SYBR Green Real-Time PCR Master Mix (Toyobo, Osaka, Japan) using a Bio-Rad CFX thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). The following sequences of specific primers were used in this study. Peroxisome proliferator-activated receptor γ (PPARγ) (forward: 5'-AAGGGTGCCAGTTTCGATCC-3', reverse: 5'-TCCTTGGCCCTCTGAGATGA-3'), adipocyte fatty acid-binding protein (aP2) (forward: 5'-GCCCAACATGATCATCAGCG-3', reverse: 5'-TGGTCGACTTTCCATCCCAC-3'), lipoprotein lipase (LPL) (forward: 5'-AACATTCCCTTCACCCTGCC-3', reverse: 5'-GTCTCTCCGGCTTTCACTCG-3'), and β-actin (forward: 5'-TACAGCTTCACCACCACAGC-3', reverse: 5'-GGAAAAGAGCCTCAGGGCAT-3'). After initial heating at 95°C for 10 s, 40 cycles of PCR (95°C for 5 s and 60°C for 31 s) were performed, followed by cooling at 4°C for 10 min. The relative expression level of genes was normalized to the β-actin level by using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Data were statistically analyzed using IBM SPSS Statistics program (version 23; IBM Corp., Armonk, NY, USA). One-way analysis of variance was followed by a post hoc Duncan's multiple comparisons test at $P < 0.05$. Data are presented as mean ± standard deviation.

Results and Discussion

KI 31-W and KI 36-W significantly reduce lipid accumulation of 3T3-L1 cells

To examine the cell viability of 3T3-L1 cells in the presence of KI31-W or KI 36-W, the cells were treated with the indicated concentrations (0, 0.09, 0.18, 0.35, and 0.7 mg/mL) of KI 31-W or KI 36-W and the cell viability was determined using MTT assay. Figure 1A and 1B indicated that bioconversion of whey by *P. pentosaceus* KI 31 or *L. sakei* KI 36 did not affect 3T3-L1 cell viability. Decreased lipid accumulation in mature 3T3-L1 cells in the presence of KI 31-W or KI 36-W was observed (Fig. 2A and 2B, respectively). A significantly reduced lipid accumulation was evident in 3T3-L1 cells treated with KI 31-W at 0.35 and 0.7 mg/mL (Fig. 2A). Likewise, lipid accumulation in 3T3-L1 cells was significantly inhibited by KI 36-W treatment at 0.18, 0.35, and 0.7 mg/mL. In particular, the lipid accumulation was reduced by over 50% following treatment with 0.7 mg/mL KI 36-W. (Fig. 2B). However, non-bioconverted whey did not reduce lipid accumulation in 3T3-L1 cells (Fig. 2C). Although whey protein and its derivatives confer health benefits against several metabolic diseases (Patel, 2015), it has not been well established that whey protein prevents adipogenesis. Enriched whey protein fractions inhibit adipogenesis of 3T3-L1 (Rajic et al., 2010). More recently, D'Souza et al. (D'Souza et al., 2020) demonstrated that whey protein hydrolyzed by pepsin and pancreatin enhances 3T3-L1 adipocyte differentiation and triacylglycerol accumulation. However, it has not yet been reported that bioconversion of whey exerts an anti-adipogenic effect. Our findings showed that

bioconversion of whey by LAB effectively inhibits adipogenesis without decreasing the proliferation of 3T3-L1 cells. Further, our observation demonstrated that, among the proteins separated by SDS-PAGE, bovine serum albumin from bioconversion of whey by LAB was decreased compared to the protein of non-bioconverted whey (Fig. 2D). It has been shown that bovine serum, which contains albumin, facilitates the proliferation and differentiation of cells (Cho et al., 2018), assuming that the decreased bovine serum albumin by the bioconversion may be associated with the reduction of adipogenic differentiation.

KI 31-W and KI 36-W down-regulate mRNA expression of genes related to adipogenesis and lipogenesis

As shown in Fig 3A, KI 31-W remarkably decreased mRNA expression of PPARγ at doses of 0.35 and 0.7 mg/mL in 3T3-L1 cells. Similar effects on the down-regulation of aP2 and LPL were seen in the cells treated with 0.35 and 0.7 mg/mL. Figure 3B demonstrated that KI 36-W also significantly down-regulated PPARγ at doses of 0.35 and 0.7 mg/mL in 3T3-L1 cells. However, aP2 mRNA expression was significantly decreased at 0.7 mg/mL KI36-W, but not at 0.35 mg/mL. In addition, LPL mRNA expression was strongly inhibited by KI 36-W at 0.09 to 0.7 mg/mL. Exposure of 3T3-L1 cells to dexamethasone, isobutylmethylxanthine, insulin, and fetal bovine serum, triggers a cascade of transcriptional activity, resulting in the rapid induction of regulators, such as PPARγ (Morrison and Farmer, 2000). Activation of PPARγ positively regulates downstream target genes, such as aP2 and LPL, associated with growth and differentiation (Farmer, 2005). Adipogenesis is strongly regulated by sequential activation of various transcription factors, such as PPARγ (Li et al., 2016). A dramatic increase in PPARγ gene expression was observed 5 days after the induction of 3T3-L1 cell differentiation (White and Stephens, 2010). The reduced expression of PPARγ was observed from an early stage (day

3) and final late-stage (day 8) during 3T3-L1 cell differentiation (Li et al., 2016). Our present study also showed that both KI 31-W and KI 36-W significantly suppressed PPARγ gene expression 9 days after the induction of differentiation. In addition, increased PPARγ expression sequentially up-regulates specific adipocyte genes, such as aP2 and LPL, to control fatty acid metabolism (Hassan et al., 2007). As a member of the fatty acid-binding proteins, aP2, which is highly expressed in mature adipocytes, plays an essential role in the intracellular fatty acid transport and metabolism critical for maintaining glucose and lipid homeostasis (Elmasri et al., 2009). LPL plays a pivotal role in the generation of fatty acids and their uptake by cells. Hence, the reduction of LPL expression during adipocyte differentiation may lead to decreased lipid storage in cells (Linehan et al., 2012).

In conclusion, we found that bioconversion of whey by LAB has the potential to suppress the adipogenic differentiation of 3T3-L1 cells, although it does not inhibit cell proliferation. Bioconversion of whey by LAB effectively and markedly reduces a key adipogenesis transcription factor, PPARγ, as well as specific lipogenesis genes, aP2 and LPL, consequently attenuating the adipogenic differentiation. To our best knowledge, this study is the first to demonstrate that bioconversion of whey has a beneficial effect.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Author Contributions

Conceptualization: Kang SS. Data curation: Lee JS, Hyun IK, Yoon JW, Seo HJ, Kang SS. Formal analysis: Lee JS, Hyun IK, Yoon JW, Seo HJ. Methodology: Yoon JW, Seo HJ, Kang SS. Software: Lee JS, Hyun IK. Validation: Lee JS, Hyun IK, Yoon JW, Seo HJ. Investigation: Lee JS, Hyun IK, Yoon JW, Seo HJ. Writing - original draft: Lee JS, Hyun IK. Writing - review & editing: Lee JS, Hyun IK, Yoon JW, Seo HJ, Kang SS.

Ethics Approval

This article does not require IRB/IACUC approval because there are no human and animal participants.

References

Asha S, Vidyavathi M. 2009. Cunninghamella--a microbial model for drug metabolism studies--A review. Biotechnol Adv 27:16-29.

Bjornshave A, Hermansen K. 2014. Effects of dairy protein and fat on the metabolic syndrome and type 2 diabetes. Rev Diabet Stud 11:153-166.

Boguta AM, Bringel F, Martinussen J, Jensen PR. 2014. Screening of lactic acid bacteria for their potential as microbial cell factories for bioconversion of lignocellulosic feedstocks. Microb Cell Fact 13:97.

Cho H, Lee A, Kim K. 2018. The effect of serum types on chondrogenic differentiation of adipose-derived stem cells. Biomater Res 22:6.

D'souza K, Mercer A, Mawhinney H, Pulinilkunnil T, Udenigwe CC, Kienesberger PC. 2020. Whey peptides stimulate differentiation and lipid metabolism in adipocytes and

ameliorate lipotoxicity-induced insulin resistance in muscle cells. Nutrients 12:425.

Domingues L, Dantas MM, Lima N, Teixeira JA. 1999. Continuous ethanol fermentation of lactose by a recombinant flocculating saccharomyces cerevisiae strain. Biotechnol Bioeng 64:692-697.

Elmasri H, Karaaslan C, Teper Y, Ghelfi E, Weng M, Ince TA, Kozakewich H, Bischoff J, Cataltepe S. 2009. Fatty acid binding protein 4 is a target of vegf and a regulator of cell proliferation in endothelial cells. FASEB J 23:3865-3873.

Farmer SR. 2005. Regulation of ppargamma activity during adipogenesis. Int J Obes (Lond) 29 Suppl 1:S13-16.

Hassan M, El Yazidi C, Landrier JF, Lairon D, Margotat A, Amiot MJ. 2007. Phloretin enhances adipocyte differentiation and adiponectin expression in 3T3-L1 cells. Biochem Biophys Res Commun 361:208-213.

Hegazy ME, Mohamed TA, Elshamy AI, Mohamed AE, Mahalel UA, Reda EH, Shaheen AM, Tawfik WA, Shahat AA, Shams KA, Abdel-Azim NS, Hammouda FM. 2015. Microbial biotransformation as a tool for drug development based on natural products from mevalonic acid pathway: A review. J Adv Res 6:17-33.

Lee NK, Paik HD. 2017. Bioconversion using lactic acid bacteria: Ginsenosides, gaba, and phenolic compounds. J Microbiol Biotechnol 27:869-877.

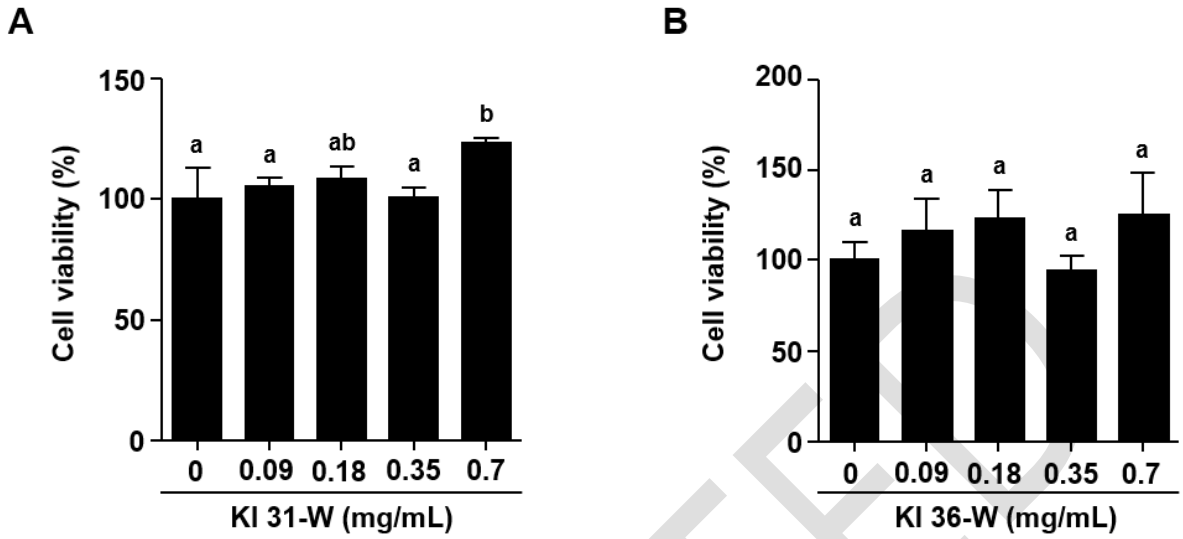
Li KK, Liu CL, Shiu HT, Wong HL, Siu WS, Zhang C, Han XQ, Ye CX, Leung PC, Ko CH. 2016. Cocoa tea (Camellia *ptilophylla*) water extract inhibits adipocyte differentiation in mouse 3T3-L1 preadipocytes. Sci Rep 6:20172.

Lin T, Lin C, Wang Y. 2002. Linoleic acid isomerase activity in enzyme extracts from *Lactobacillus acidophilus* and *Propionibacterium freudenreichii* ssp. *Shermanii*. J Food Sci 67:1502-1505.

242 Linehan C, Gupta S, Samali A, O'connor L. 2012. Bisphenol A-mediated suppression of LPL
243 gene expression inhibits triglyceride accumulation during adipogenic differentiation of
244 human adult stem cells. PLoS One 7:e36109.
245 Marshall K. 2004. Therapeutic applications of whey protein. Altern Med Rev 9:136-156.
246 Morrison RF, Farmer SR. 2000. Hormonal signaling and transcriptional control of adipocyte
247 differentiation. J Nutr 130:3116S-3121S.
248 Patel S. 2015. Emerging trends in nutraceutical applications of whey protein and its derivatives.
249 J Food Sci Technol 52:6847-6858.
250 Rajic A, Dhulia J, Hosking CG, Autelitano DJ. 2010. A novel dairy-derived isolate that inhibits
251 adipogenesis and significantly reduces weight gain in a high fat animal model. Int Dairy
252 J 20:480-486.
253 Smitha M, Singh S, Singh R. 2017. Microbial biotransformation: A process for chemical
254 alterations. J Bacteriol Mycol Open Access 4:00085.
255 Sornplang P, Piyadeatsoontorn S. 2016. Probiotic isolates from unconventional sources: A
256 review. J Anim Sci Technol 58:26.
257 Spiegelman BM, Flier JS. 2001. Obesity and the regulation of energy balance. Cell 104:531-
258 543.
259 Walzem RL, Dillard CJ, German JB. 2002. Whey components: Millennia of evolution create
260 functionalities for mammalian nutrition: What we know and what we may be
261 overlooking. Crit Rev Food Sci Nutr 42:353-375.
262 White UA, Stephens JM. 2010. Transcriptional factors that promote formation of white adipose
263 tissue. Mol Cell Endocrinol 318:10-14.
264 Yang H, Lee SH, Ji H, Kim JE, Yoo R, Kim JH, Suk S, Huh CS, Park JHY, Heo YS, Shin HS,
265 Kim BG, Lee KW. 2019. Orobol, an enzyme-convertible product of genistein, exerts

anti-obesity effects by targeting casein kinase 1 epsilon. Sci Rep 9:8942.

290 **Figure legends**



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293 **Fig. 1.** Cell viability of 3T3-L1 adipocytes in the presence of KI 31-W and KI 36-W. The cell

294 viability was determined by MTT assay after 3T3-L1 cells were treated with KI 31-W or KI 36-

295 W for 24 h. The results were obtained from three independent experiments.

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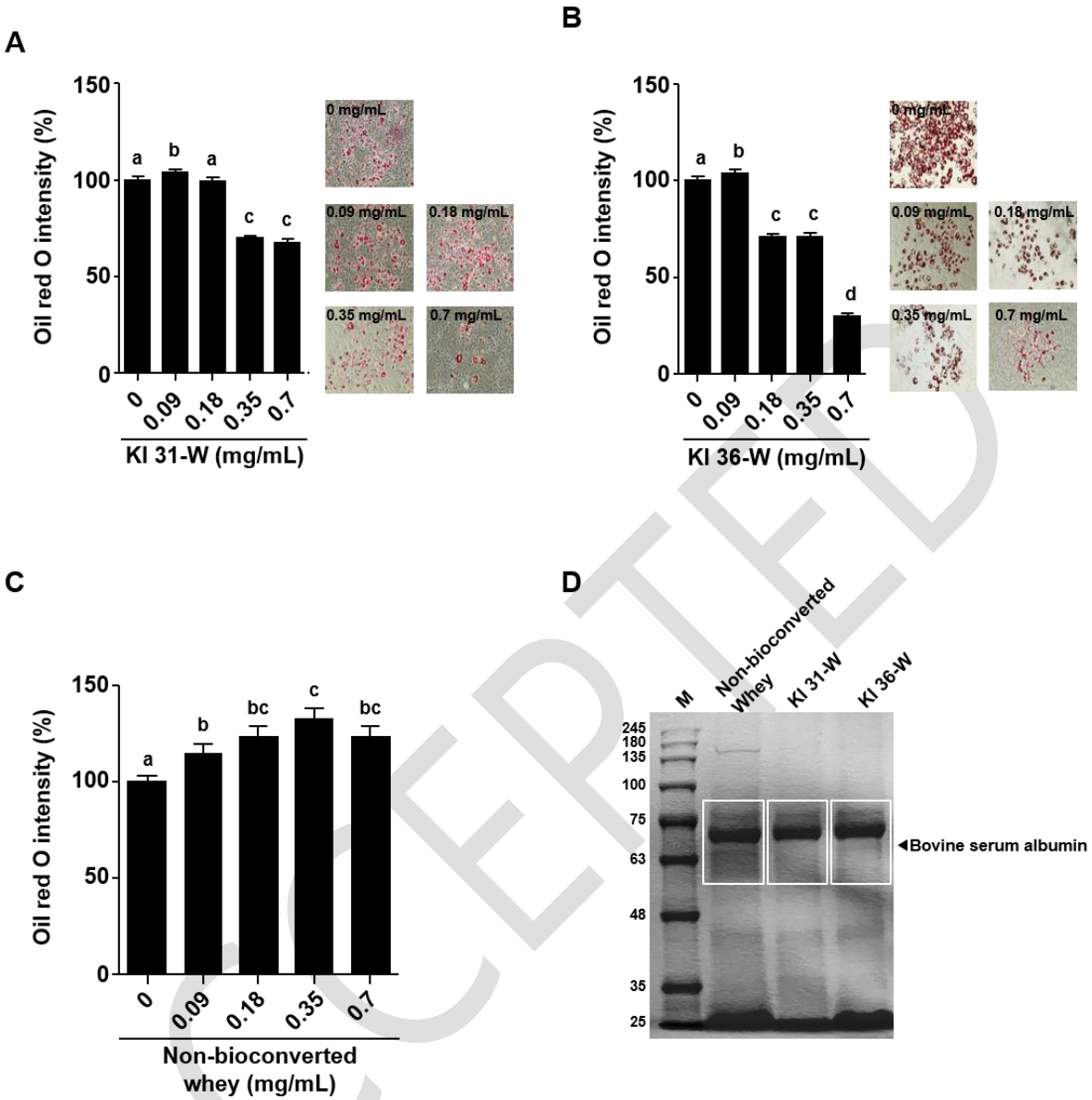
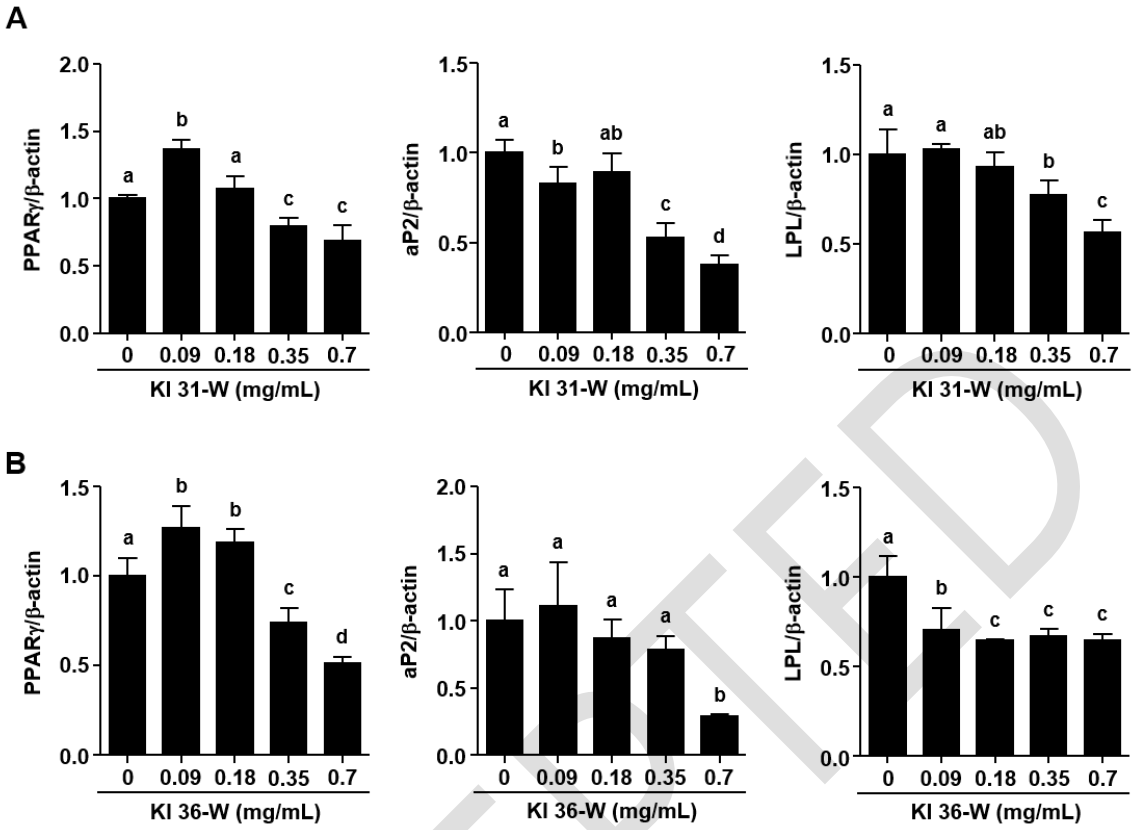


Fig. 2. Effect of KI 31-W and KI 36-W on lipid accumulation in 3T3-L1 adipocytes. Determination of lipid content by Oil Red O staining in fully differentiated 3T3-L1 adipocytes treated with KI 31-W (**A**) or KI 36-W (**B**). Images represent microscopy of Oil Red O-stained cells. The results were obtained from three independent experiments. **(C)** lipid accumulation in 3T3-L1 cells in the presence of non-bioconverted whey was determined by Oil Red O staining. **(D)** protein profiles of non-bioconverted and bioconversion of whey were analyzed by SDS-PAGE. M: size marker.



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306 **Fig. 3.** Effect of KI 31-W and KI 36-W on the mRNA levels of adipogenesis- and lipogenesis-
307 related genes in 3T3-L1 adipocytes. The mRNA expression of PPARγ, aP2, and LPL in 3T3-
308 L1 adipocytes in the presence of KI 31-W or KI 36-W were quantified by real-time RT-PCR
309 analysis. The results were obtained from three independent experiments.

310