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Original Article

A novel approach towards obesity: The use of a bacterial product, gassericin A, in 3T3-L1 cells

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ABSTRACT

Background and objective: The problem of obesity and its related complications are adversely affecting human society. We studied the effects of gassericin A, a bacteriocin produced by the intestinal bacteria, on adipocyte differentiation and development.
Design: Gassericin A was purified from *Lactobacillus gasseri* LA39 and was added to the culture medium of 3T3-L1 cells in two phases: Phase 1, 3T3-L1 cells were incubated with gassericin A while being induced to adipocytes (days 1–7); phase 2, the cells were incubated with the bacteriocin after being induced to adipocytes (days 8–12). The resultant changes in the pattern of expression of some of the important genes involved in adipogenesis were evaluated by RT-qPCR. The viability of cells and their numbers were also studied.
Results: In phase 1 of the study, the levels of transcripts for stearoyl CoA desaturase (SCD-1), zinc finger protein 423 (zfp-423), and glucose transporter 4 (GLUT4) genes were significantly reduced, while that of 422ap2 gene showed a significant increment ($p < 0.05$). In phase 2, the zfp-423 gene showed a reduction of expression and the 422ap2 gene showed an increase in expression ($p < 0.05$). The other genes including UCP-1 and TNF-α did not show any significant changes in neither of the groups. Gassericin A did not affect the morphology or viability of the cells, however, the numbers of cells had nearly doubled in the treatment groups.
Conclusion: It seems that gassericin A could significantly alter the properties of adipocytes while they are in the process of development and after they have developed.

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Introduction

Overweight and obesity are defined as the excessive accumulation of fat in the body. According to the WHO fact-sheet 2016, obesity has tripled since 1975, with nearly 40% of people of 18 years old and higher being affected by overweight and 13% with obesity. Another study suggests the problem of overweight affecting children; 40 million kids under the age of five were affected by obesity in 2018 [1].

The disorders related to obesity are various and quite severe. Type 2 diabetes and the complications related to it are closely associated with obesity. About 90% of people with type 2 diabetes are also affected by obesity [2]. Cardiovascular diseases are among other causes of ill health accompanying obesity, including coronary artery disease and hypertension [3]. A variety of malignancies such as those of the colon, pancreas, and liver are also connected with obesity. The latest data suggest that for each BMI increase equal to 5 kg/m^2, the risk of cancer development is also increased by 1.6 times [4].

There are nearly 10^{14} microbial cells in the human gastrointestinal tract, and the amount of genetic material in these microbial cells is approximately one hundred times as much as the human genome size [5]. These microorganisms assume various and influential roles, from modulation of immunity to harvesting food energy to weight. Experiments have suggested that transferring fecal material between individuals, termed fecal microbial transplant (FMT), can modulate the host weight and metabolic status [6]. Lipopolysaccharides have been shown to reduce adipogenesis in preadipocyte 3T3-L1 cells [7]. However, there is evidence that the increase in circulating LPS could lead to obesity and adipocyte decrepitude [8].

Bacteriocins are small, low molecular weight peptides that are synthesized in bacterial ribosomes. Bacteriocins possess antimicrobial

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properties [9]. An important group of human intestinal microbiota, the *Lactobacillus* genus, produces a wide variety of bacteriocins such as gassericin A, reutericin, plantaricin, colicin, nukacin, salivaricin, and pediocin. Based on bioinformatics analyses, these peptides might show some weight change effects: some cause an increase and some might cause a decrease in adiposity and weight [10]. However, these speculations have not been verified using *in vivo* or *in vitro* experiments. Gassericin A of *Lactobacillus gasseri* LA39 is a 58 amino acid cyclic peptide whose molecular weight is 5652 and whose primary structure contains the sequence IYWIADQFGIHLATGTARKLLDA MASGASLGTAFAAI LGVTLPAWALAAAGALGATA [11]. This bacteriocin has been categorized among weight-protective bacteriocins, *i.e.* it might be effective in reducing weight [10]. Based on this, we used this bacteriocin in our research to see whether we could verify any relations between this peptide and the metabolic state in adipose tissue.

Materials and methods

The bacteriocin producer species, *L. gasseri* LA39 (IBRC, Iran), was purchased from the Iranian Biological Research Center (IBRC, Iran) in the form of active culture. The indicator species, *L. delbrueckii* subsp. *bulgaricus* was also purchased in the form of lyophilized powder (IBRC, Iran).

The purification of bacteriocin was performed according to Kawai et al., with moderate modifications [12]. Briefly, *L. gasseri* was cultured in MRS broth medium (Merck Inc., Germany) for 38 h at 37 °C. To purify the bacteriocin, a modified MRS broth, namely DOB-MRS broth (all components from Sigma-Aldrich Co., USA or Germany), was prepared. All the components of MRS-broth except Tween 80 and beef extract were dialyzed against water and then 0.1% (v/v) of oleic acid was added to the diffusate. The *L. gasseri* LA39 was cultured in DOB-MRS broth for 48 h at 37 °C; then, the supernatant was obtained following centrifugation (4 °C, g = 5000) for 20 min. Oleic acid was separated by filter paper, and the filtrate was dialyzed against water (Sigma dialysis tube D7884-1FT, MWCO: 2000, Germany). In the next step, the dialysate was taken to a hydrophobic chromatography column (TOYOPEARL Butyl-650 S, TOSOH, Japan) and was subjected to elution by distilled water (this fraction was gathered for the next chromatography column), 50% (v/v) and then 100% methanol. The eluate with water was taken to reverse phase chromatography (LiChroprep RP-8, Germany), and was washed by water, 20% acetonitrile, and 20%, 40%, 60%, and 90% 2-propanol in water, respectively. The eluate of 60% 2-propanol (the working fraction) was kept at −20 °C for further processing. In the next step, the working fraction was subjected to lyophilization (IlShin Freeze Dryer, IlShin BioBase, the Netherlands). The samples were dried after 12 h of freeze–drying and were dissolved in saline solution. The final solution was aliquoted and kept at −80 °C until the cellular experiments. The final concentration of bacteriocin was 4 units (arbitrary) per aliquot.

The bacteriocin was assayed by soft MRS agar well diffusion by adding 0.7% agar to MRS broth in 4 mm deep plates. The plates were cultured with the indicator species, *L. delbrueckii bulgaricus*. Wells of 5 mm in diameter were cut out of the agar. Using two-fold serial dilutions, different concentrations of the bacteriocin were prepared. The highest dilution that inhibited the growth of the indicator bacterium was arbitrarily defined as one unit [13].

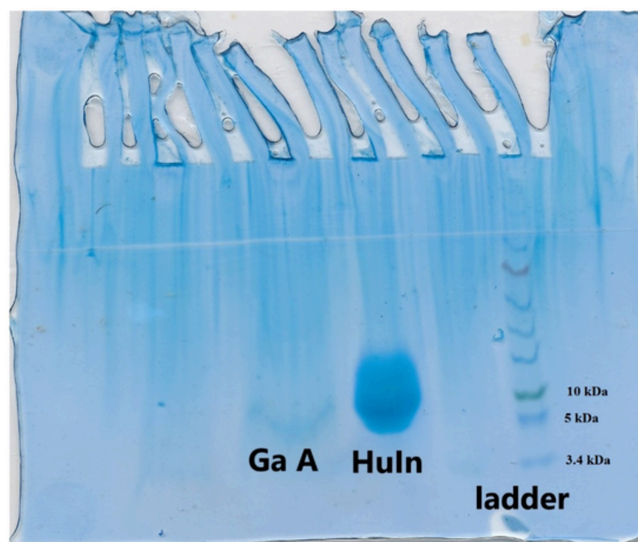

Fig. 1. Tricine-SDS-PAGE showing gassericin A. Ga A: gassericin A, HuIn: human insulin. The ladder shows peptides as small as 3.4 kDa. Gassericin A can be seen at around 5 kDa.

To verify the purification of the peptide, tricine-SDS-PAGE was done according to the Schagger method [14]. A protein ladder (CinnaGen, Iran) was used to indicate the peptide; however, we also applied human insulin which was roughly the same size as gassericin A (Fig. 1).

Cellular experiments

3T3-L1 cells (IBRC, Iran) were cultured in high glucose DMEM (Gibco, USA) with 10% fetal bovine serum (DENA Zist Asia, Iran) and 1% penicillin–streptomycin (Sigma, Germany). The cells were kept at 37 °C with 5% CO_2 by changing the culture medium every other day until two days after they had reached confluence (day 0); from day 0 to day 2 the culture medium was changed daily and it was supplemented with 3 nM of human insulin (Sigma, Germany), 0.25 μM dexamethasone (Sigma, Germany), and 0.5 mM 1-methyl-3-isobutyl-xanthine (Sigma, Germany). From day 3 to 7, the culture medium was just supplemented with 3 nM of insulin [15]. The study was designed in two phases with three repetitions in each phase: in phase 1, 3T3-L1 cells were incubated with gassericin A (1×10^{-2} μ ml^{-1}) from day 0 to 7, while in phase 2 the cells were incubated with the bacteriocin from day 8 to 12, *i.e.* five days after the induction process had finished (Fig. 2). The concentration of gassericin A was chosen based on pilot studies. While using the bacteriocin, the cells showed strong proliferation and gathered in the form of spheres on one side of the plate. The lowest concentration which induced this kind of gathering (1×10^{-2} μ ml^{-1}) and did not exert apparent toxic effects was chosen for the treatment. This concentration was not toxic as verified by the lack of detached or dead cells, but it was an effective concentration since the cells were proliferating. Each phase had its control group which was only induced and received no treatment. We treated 3T3-L1 cells in two phases: 1. While the process of induction to see whether gassericin A could prevent or accelerate the process and affect the number and genetic profile of the

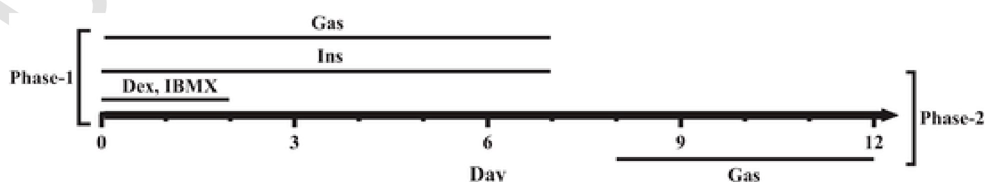

Fig. 2. The process of induction and treatment in two phases of the study. Gas, gassericin A; Ins, insulin Dex: dexamethasone; IBMX, 1-methyl-3-isobutyl-xanthine.

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preadipocytes compared to the control group; 2. After the process of induction had finished [15] to see whether gassericin A has any effects on mature adipocytes. At the end of the treatment process, the general morphology of cells was assessed using an inverted microscope, and cell counting was achieved using a hemocytometer. Trypan blue staining was used to check the cell viability changes.

The ratios of gene expression in genes of interest were determined by qPCR (BIO-RAD, USA) and the data were analyzed by the relative Pfaffl method [16]. To avoid any DNA contamination affecting the results, exon–exon primers were used (Table 1). The genes of interest were the following: TATA-binding protein (TBP), uncoupling protein 1 (UCP-1), zinc finger protein 423 (zfp423), fatty acid-binding protein 4 (FABP4, also known as 422ap2), tumor necrosis factor alpha (TNF-α), stearoyl-CoA desaturase 1 (SCD-1), and glucose transporter type 4 (GLUT4). It is noteworthy that TBP was used as the reference gene in qPCR. It is the most stable gene in white and brown adipose tissue in mice [17]. The binding of this protein to TATA box is necessary for the transcription of many eukaryotic genes [18].

Table 1
Primers used in the amplification of genes in RT-qPCR.

Gene	Forward primer	Reverse primer
422ap2	TGAAATCACCGCAGACGACA	ACACATTCCACCACCAGCTT
GLUT4	GCCCGGACCCTATACCCTAT	GGGTTCCCCATCGTCAGAG
SCD-1	CAGGTTTCCAAGCGCAGTTC	ACTGGAGATCTCTTGGAGCA
TBP	CCTATCACTCCTGCCACACC	ATGACTGCAGCAAATCGCTTG
TNF-α	TAGCCCACGTCGTAGCAAAC	GCAGCCTTGTCCCTTGAAGA
UCP-1	TGGAAAGGGACGACCCCTAA	CAGGAGTGTGGTGCAAAACC
Zfp-423	CCGCGATCGGTGAAAGTTGA	ACGCTGTTCCTGTCTTCCAG

Unless otherwise stated, all results are mentioned as median and quartiles. Regarding the skewed distribution of some data sets, the non-parametric Mann–Whitney test was used to compare the treatment group with the control in the same phase of the experiment. In all cases, $p < 0.05$ was considered statistically significant.

Results

Gassericin A did not affect the general morphology of cells during adipogenesis or in the differentiated 3T3-L1 cells, although the group that received gassericin A while getting induced to adipocytes showed fewer fat droplets and more hollow spaces among and inside the cells (Fig. 3). The viability of the cells in trypan blue staining did not show any significant differences. However, the peptide caused a significant rise of 1.8–2 times in the number of cells in both phases of the study. The gene expression of UCP-1 did not change due to gassericin A in either of the experiments; nevertheless, in pre-adipocytes (phase 1 of the study), there were significant changes in the levels of expression of other genes of interest: the expression level of SCD-1 showed an important reduction relative to the control group ($p = 0.0379$); the same was true about zfp423 ($p = 0.0005$) and GLUT4 ($p < 0.0001$); while the level of expression of 422ap2 showed a significant increase in the treatment group relative to the control ($p = 0.0106$). TNF-α was not de-

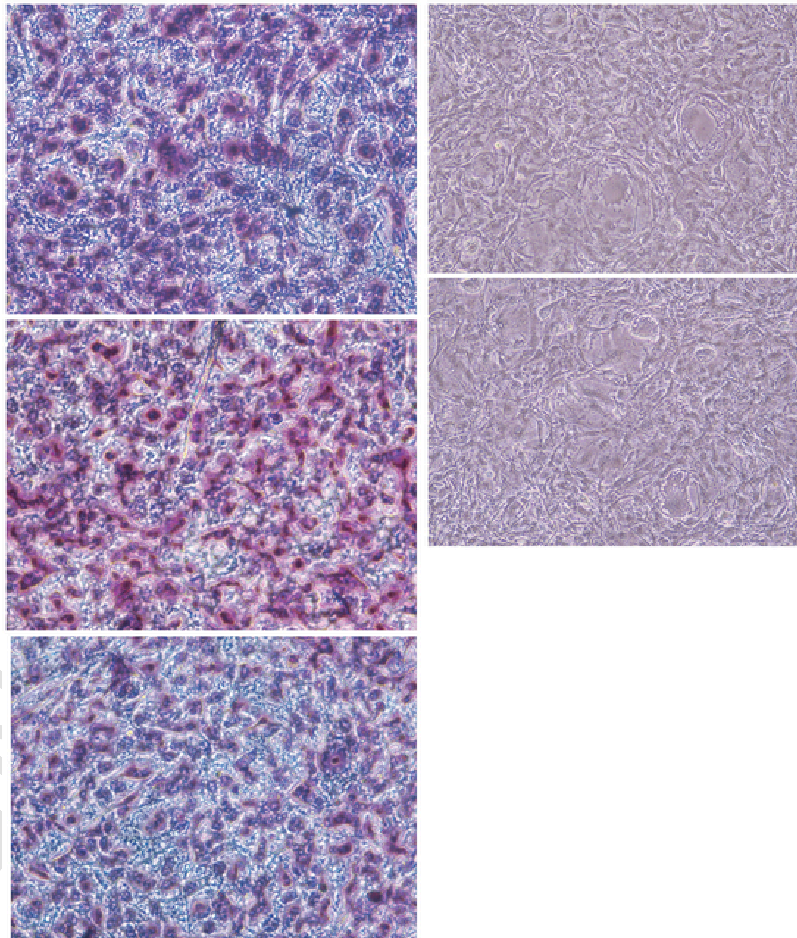

Fig. 3. 3T3-L1 cells. First: oil-red-o staining non-induced cells, second: oil-red-o staining induced cells without gassericin A showing red stained fat inside the cells. Third: oil-red-o staining induced cells with gassericin A during induction; a considerable reduction of fat content and red stain density can be seen. Fourth: no staining inverted microscope, the control group (induced, no gassericin A), last: no staining inverted microscope, with gassericin A during induction (inverted microscope, ×200).

tectable in these cells. In differentiated adipocytes (phase 2 of the study), there was a significant increase in the expression of 422ap2 in the treatment group relative to the control group ($p < 0.0001$) and a significant decrease in the expression level of zfp423 ($p < 0.0001$). The other genes did not show significant changes in gassericin A-treated cells compared to the control (Fig. 4).

Discussion

There is a growing body of evidence suggesting a pivotal role for intestinal microbiota in the onset and progression of obesity, and bacteriocins are indispensable to the niche of bacteria in the gastrointestinal tract [19]. We chose a bacteriocin from an important member of the genus *Lactobacillus* to see whether it can exert modifications during the process of adipogenesis or in differentiated 3T3-L1 cells. Different species of *Lactobacillus* including *L. gasseri* have been successfully used in animal models of obesity [3]. We initially verified the possible effects of gassericin A on the size and morphology of the cells. There is no data regarding the bioavailability of gassericin A, and therefore it is unclear to what extent the peptide may escape gastrointestinal digestion and whether it can be absorbed from the intestinal barrier.

In this study, gassericin A did not cause detectable changes in morphology or viability of the cells, however, it nearly doubled the number of cells in both phases of the study. The adipose tissue has an aspect of metabolic and energy buffering, and the two ways of performing this duty are by hypertrophy (the increase in the size of the cells) and hyperplasia (the increase in the number of the cells). However, when a hypertrophic threshold is outstripped and hyperplasia does not occur, the hypertrophy changes to a state of fibrosis and its pursuing and conflicting metabolic alterations. It has been shown that adipocyte hypertrophy could be associated with hypoxia. In addition, when pre-adipocyte hyperplasia does not happen, the chance of developing metabolic problems broadens [20]. Previously it was thought that the number of adipocytes was determined early in life. The recent data suggest that in the time of caloric excess, new adipocytes can emerge from pre-adipocytes. The small adipocytes confer more immunity against metabolic conditions such as diabetes. As they become larger and hypertrophic, the cell becomes subjected to mechanical and hypoxic stress which can lead to adipose tissue inflammation. Larger adipocytes show more lipolysis, increased secretion of inflammatory cytokines, and decreased secretion of anti-inflammatory adipokines such as adiponectin [21]. Consistently, the inhibitors of S-phase cell cycle promoters such as S-phase kinase-associated proteins (Skp) cause resistance to obesity via inhibition of pre-adipocyte proliferation without causing adipocyte hypertrophy; however, this is not the unique effect imposed by their inhibition since Skp2 deficient mice decline the number of pancreatic β cells [22]. These facts suggest the possibility that gassericin A might exert protective effects against the problems caused by insufficient hyperplasia of adipose tissue since it causes an increment of cell numbers both before and after differentiation. However, this bacteriocin might exert negative consequences in a living animal model such as hyperplastic obesity. Therefore, animal tests are essential before any solemn thesis can be sentenced.

Stearoyl-CoA desaturase-1 (SCD-1 or Δ-9-desaturase) is an endoplasmic reticulum enzyme that catalyzes the rate-limiting step in the production of monounsaturated fatty acids (MUFA), especially the conversion of palmitic and stearic acid into palmitoleic and oleic acid. The expression of SCD-1 is markedly upregulated during adipocyte differentiation. The decrease in SCD-1 reduces the levels of palmitoleic and oleic acid, and this is followed by a reduction of triacylglycerol synthesis sometimes up to one-third [23]. The products of SCD-1, *i.e.* MUFA, are required for the synthesis of triglycerides, cholesterol esters, and very-low-density lipoprotein (VLDL). When SCD-1 is downregulated, saturated fatty acyl CoAs cannot be converted into MUFAs; so they accumulate in the cytosol. Saturated fatty acyl CoA allosterically inhibits acetyl CoA carboxylase, which leads to a reduction in the conversion of acetyl CoA to malonyl CoA. The reduction in the amount of malonyl CoA removes the allosteric inhibition of carnitine palmitoyltransferase 1. The latter enzyme catalyzes the shuttling of fatty acids from the cytosol to mitochondria where they are oxidized. Therefore, a reduction in SCD-1 leads to an increase in fatty acid oxidation [24]. Previous studies suggest that reduced SCD-1 activity is followed by improved insulin sensitivity and a reduction in body weight. Downregulation of SCD-1 (*via* leptin) causes the reduction of fat in the liver. Downregulation of SCD-1 in mice leads to both losses of weight and amelioration of hepatic steatosis. Downregulation of SCD-1 has also been associated with reduced cancer cell growth in the lung, breast, colon, and prostate. In many cancer types, *e.g.* colon, esophagus, and liver malignancies, an increase in SCD-1 expression has been observed which has been attributed to augmented energy requirement in these cells [25]. Using isolated adipocytes, SCD-1 deficient cells show reduced inflammatory reaction to treatment with lipopolysaccharide, although some minor side-effects occurred [26]. In this study, gassericin A caused a significant decrease in the expression of SCD-1 gene in differentiating preadipocytes. This reduction might be associated with weight loss and decreased obesity-related complications.

The expression of 422ap2 (fatty acid-binding protein 4) was also increased in both phases of the study. This gene is one of the two main identifiers of adipocytes with the other being SCD-1. Normally these genes go together [27], however, our results do not show this, making it impossible to conclude whether gassericin A stimulates differentiation of the cells toward mature adipocytes. According to Oil Red O staining results, gassericin A caused less fat accumulation within the cells, but the reason remains obscure because of the possible intervention made by the browning of white adipocytes. The expression of 422ap2 gene is augmented during the differentiation of pre-adipocytes to adipocytes [28] and is downregulated by treatment with lipopolysaccharide [7]. The protein expressed by 422ap2 gene is a fatty acid-binding protein (FABP). These proteins are involved in the transport of saturated and unsaturated fatty acids as well as eicosanoids, leukotrienes, prostaglandins, and other lipids to different cellular compartments such as mitochondria and lipid droplets. This protein is also expressed in macrophages and has an important role in atherosclerosis; however, the source of artery lesions is the expression of ap2 in macrophages only, and not adipose tissue [29]. Mice lacking adipocyte FABP4 (ap2) and FABP5 (mal1) show more protection against obesity, diabetes type 2, insulin resistance, and fatty liver disease, as well as reduced liver expression of SCD-1 [30]. The discrepancy observed in our results demands further studies employing other specific markers of mature adipocytes.

The other gene of interest, GLUT4, displayed a significant decrease of expression in phase 1 of the study, but no changes in phase 2. Glucose transporter type 4 (GLUT4) is the main transporter of glucose into adipocytes. It lies in the membrane of adipocytes, skeletal, and cardiac myocytes and facilitates the transport of glucose into these cells by facilitated transport. It is an insulin-responsive transporter, and its reduction in adipocytes of individuals with obesity is a primary sign of the onset of insulin resistance [31]. However, the overexpression of GLUT4 in mice is associated with adiposity and increased serum free fatty acid (FFA) levels [32]. Although gassericin A reduced GLUT4 expression in the pre-adipocyte state (phase 1 of the study), there is a need to evaluate the fate of the cells *in vivo* to see whether this decrease will continue in the adult tissue.

Most mammalian species develop two types of adipocytes which specialize in dissipating heat through UCP-1 to protect the animal against hypothermia, which are termed brown and beige (brite) cells. These cells express considerable amounts of UCP-1 which transforms the energy of protons to heat instead of ATP production. While brown cells have their specific depots, beige cells are interspersed among white adipocytes. The expression of UCP-1 is augmented in brown and

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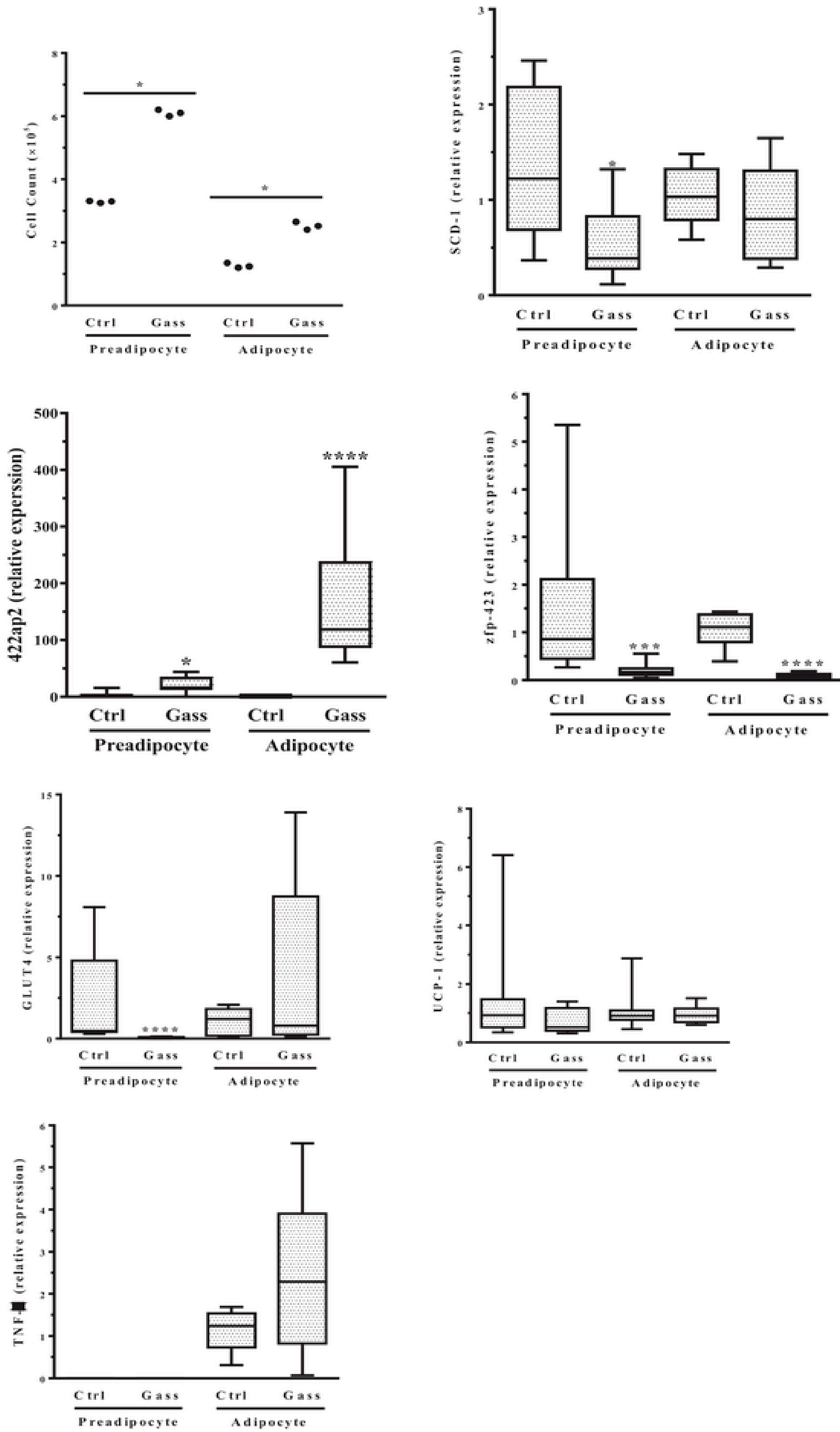
Cell Count (×10⁵)
SCD-1 (relative expression)
422ap2 (relative expression)
zfp-423 (relative expression)
GLUT4 (relative expression)
UCP-1 (relative expression)
TNF (relative expression)
Ctrl
Gass
Preadipocyte
Adipocyte

◀ **Fig. 4.** The effects of gassericin A on the expression of some genes associated with adipocyte metabolism during the differentiation of preadipocytes, 3T3-L1 cells, and in mature fat cells. Adipogenesis was induced on day 0 and the cells were treated with gassericin A either during differentiation period (days 0–7, left) or following maturation (days 8–12, right), and then RNA extraction was done for RT-qPCR on day 8 for phase 1 and on day 13 for phase 2 of the study. UCP-1: uncoupling protein 1, SCD-1: stearoyl CoA desaturase-1, zfp423: zinc finger protein 423, 422ap2: a fatty acid-binding protein, GLUT4: glucose transporter 4. Data are represented as median and quartiles. The asterisks represent statistical significance (*: $p < 0.05$, ***: $p < 0.001$, ****: $p < 0.0001$, Mann–Whitney test).

beige adipocytes and free fatty acids are the main fuel molecules for activating thermogenesis through UCP-1; therefore, activating UCP-1 could lead to a reduction in fat mass and consequently body weight [33]. Since 3T3-L1 cells have the properties of preadipocytes and they have shown the expression of UCP-1 in previous studies [15], we evaluated the level of UCP-1 in 3T3-L1 cells in preadipocyte and adipocyte stages to see whether gassericin A could induce browning in these cells. Although the bacteriocin had no detectable effects on the expression of UCP-1 in either phase of the study, another gene encoding zfp423, a zinc finger protein that is essential for maintaining the identity of white adipocytes, showed a dramatic reduction in both phases of the study. White adipose tissue (WAT) carries the burden of energy storage, and despite having many of the transcription factors of brown and beige tissue, it resists the program of heat production. One key transcriptional regulator of WAT is zfp423 which regulates the levels of PPARγ in preadipocyte mural cells and commits them to adipogenesis. It is also present in mature adipocytes of WAT. This protein suppresses the thermogenic program in WAT by suppressing the activity of Ebf2, which along with Prdm is a transcription factor for brown/beige lineage commitment. Genetic ablation of zfp423 in white adipocytes leads to the appearance of beige characteristics. On the contrary, the elevation of zfp423 expression in brown adipocytes leads to the appearance of white adipocyte characteristics [34]. Based on previous studies, lipopolysaccharide could reduce the total amount of lipid in adipocytes, and one of the genes suppressed by LPS is zfp423 [8]. In conclusion, downregulation of zfp423 in 3T3-L1 cells due to gassericin A (as seen in phase 1 of this study) might reduce the total size of WAT, while its reduction in mature adipocytes (phase 2 of the study) might start brown/beige characteristics. The latter effect was not confirmed by UCP-1 results, however, since zfp423 is an essential part of the white adipocyte genetic program, we could conclude that the cells have deviated from the path of white adipose tissue development, at least genetically. Long-term exposure to gassericin A might show some significant changes in UCP-1; nevertheless, further studies are required in this regard.

New findings suggest a link between obesity and inflammation. We, therefore, assessed the gene expression of the proinflammatory cytokine, TNF-α, in our study. The protein is increased in obesity through production by both M1 macrophages and adipocytes and leads to adipose tissue inflammation. Chronic inflammation might lead to various complications which are associated with obesity [35]. Dyslipidaemia and insulin resistance are closely associated with the expression of TNF-α in adipose tissue. This cytokine has a variety of effects ranging from inhibition of carbohydrate metabolism to low-level inflammation, obesity, and type II diabetes. Normally, the circulating level of TNF-α is low and it increases in metabolic disorders although its level in serum is correlated with the BMI. In the current study, TNF-α was not detectable during adipogenesis in either of the two groups. The cytokine was expressed in mature adipocytes, however, gassericin A did not affect its level of expression.

Conclusion

For the first time, to the best of our knowledge, we analyzed the effects of a bacteriocin from an important human gut probiotic, *L. gasseri* LA39 on the expression of the most noticeable genes in adipogenesis. Gassericin A caused hyperplasia and downregulated SCD-1 and zfp423 in treated cells, suggesting potential protective effects against obesity and its complications. However, this study had several limitations. One of the main shortcomings was the lack of previous studies on this topic. Besides, since gassericin A is not commercially available, and its extraction and purification are time-consuming and expensive, we had limited access to gassericin A. The safety of gassericin A should be studied. Moreover, several other genes involved in adipocyte differentiation such as PPARγ and C/EBPa should be also studied. In addition, the results of this research should be confirmed by *in vivo* studies. However, due to the complications caused by the possible effect of the peptide on intestinal microbiota, and also the production of the peptide by gut bacteria, the bacteriocin should be tested on germ-free animals.

Ethics

The authors declare that nowhere in this research, no human or animal subject was engaged and all the experiments were done on cell culture.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgment

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