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Mimicking Wagyu beef fat in cultured meat: Progress in edible bovine adipose tissue production with controllable fatty acid composition

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ABSTRACT

Since the current process of livestock meat production has considerable effects on the global environment, leading to high emissions of greenhouse gases, cultured meat has recently attracted attention as a suitable alternative way to acquire animal proteins. However, while most published studies on cell-cultured meat have focused on muscle tissue culture, fat production which is an important component of the process has often been neglected from this technology, even though it can enhance the meat's final taste, aroma, tenderness, texture, and palatability. In this study, we focused on bovine muscle reconstruction by monitoring and optimizing the possible expansion rate of isolated primary bovine adipose stem cells and their adipogenesis differentiation to be fully edible for cultured meat application.

After approximatively 100 days of serial passages, the bovine adipose-derived stem cells, isolated from muscle tissue, underwent 57 ± 5 doublings in the edible cell culture medium condition. This implies that by cultivating and amplifying them, a minimum of 2.9×10^{22} cells can be obtained from around 10 g of fat. It was discovered that these cells retain their adipogenesis differentiation ability for at least 12 passages. Moreover, the final lipid composition could be controlled by adjusting the fatty acid composition of the culture medium during the differentiation process, resulting in organoleptic features similar to those of real fat from muscle. This was especially so for the cis isomer oleic acid percentage, an important part of high-grade Japanese Wagyu meat.

These characteristics of the primary bovine adipose-derived stem cell proliferation and adipogenesis differentiation provide valuable insights for the *in vitro* production of meat alternatives.

1. Introduction

Cultured meat is a recent emerging technology that uses cellular agriculture of cells derived from meat animal species and tissue engineering techniques to recreate meat without sacrificing the animal. As such, this technology has several potential benefits such as eliminating animal slaughter, reducing agricultural land use, facilitating pathogen control, decreasing dependence on antibiotics and reducing environmental pollution [1].

Most experimental and theoretical work on cultured meat has until now focused mainly on muscle tissue reconstruction [2–4]. Despite this, although the main constituent of meat is commonly considered to be muscle, some types are known for their marbled structure such as Japanese Wagyu which contains more than 50% fat [5]. This meat fat is a key determinant of taste, texture, smell, nutritional value and visual appearance, but is a factor that has often been overlooked [6]. These characteristics are highly correlated with consumer preference and the incorporation of biomanufactured fat into cell cultured meat products is

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essential to ensure favorable consumer responses and commercial success.

Cultured fat cells (adipocytes) are therefore a possible answer to the problem of improving the organoleptic properties of alternative meats in a customizable and scalable manner. However, this field is still in its infancy and few sources of adipogenic cell ruminant species have hitherto been evaluated for growth and differentiation to maturation, or even used in a 3D tissue construct [7]. Most studies have been performed on human and rodent-derived cells, and adaptations of techniques to meat animal origin cells are needed to further develop cultured fat. There is also a lack of research on how adipocytes from agricultural species are ultimately isolated, produced, and incorporated as food constituents.

The fat content of foods can come either from added lipid (fatty acid molecules) or lipids contained in adipocytes (fat cells). Both are very different in terms of their biological and dietary properties, adipocytes being a natural component of animal fat, able to store large amounts of lipids [7]. Even though their final composition is more difficult to control than the direct addition of lipids to the final meat product, the use of adipocytes in food products is a largely unexplored avenue for providing alternative meat fat with a superior nutritional profile and authentic food properties. Indeed, animal fat is often perceived as undesirable because of its relatively high levels of saturated fatty acids [8]. These are generally associated with a higher risk of metabolic disorders compared to plants and fish where levels of unsaturated fatty acids are higher and have health benefits [9]. One of the assets of cultured meat production is the ability to control the saturated fat levels by adjusting the components of the culture media. It should therefore be possible to manufacture cultured fat similar to real meat fat, without compromising its overall palatability.

To speed up the process of meat production in the field of cultured meat, several attempts have been made to imitate the fat of animal meat with oil of plant origin [7]. When it comes to comparing animal fat and oil usually extracted from plants, animal products actually have a diverse dietary profile, while oil has relatively homogeneous physical characteristics and chemical composition in comparison (e.g., low melting point, 'resulting in limited flavor and nutritional value) [10]. It has been shown that the alternative use of vegetable oil is difficult, especially for maintaining the structural and functional integrity of the meat. Heavy processing is usually required, which may alter the final texture of the produced meat [11]. In contrast, adipocytes naturally store lipids within the meat tissue in a stable manner, allowing the taste, texture and appearance of animal meat to be mimicked. For these reasons, cultured adipocyte tissue appears to have greater potential for customizing the quality of alternative meat.

So far, only a few published reports have described adipogenic protocols that specifically use bovine cells from the viewpoint of cultivated meat [12–16]. To address this, we focused herein on analyzing the expandability of bovine adipose-derived stem cells (ADSC), in food-grade culture, until the adipocytes reached a mature state. The objective was to provide scalable protocols for edible adipose tissue construction. Actually, the safety of some of the components commonly used for adipogenesis in human or rodent cells remains uncertain. Adipogenesis is most often achieved using a three-component hormonal cocktail of insulin, dexamethasone, and isobutylmethylxanthine (IBMX), which can be problematic for cultured meat product application due to the acute oral toxicity of IBMX [17] or the steroidal nature of dexamethasone [18]. Fortunately, bovine preadipocyte differentiation strategies have recently begun to use other approaches. These include induction by direct addition of free fatty acids, which is the method we used, designed specifically to meet the needs of cellular agriculture [15,16].

Finally, as the organoleptic composition is of importance for cultured meat, and is often overlooked in published studies, the exact final lipid composition was assessed and compared to real bovine meat fat.

2. Results and discussion

2.1. Cell isolation and proliferation assessment

The initial stage of cellular agriculture involves the isolation of primary cell from meat species tissues, followed by their subsequent expansion and differentiation for the construction of meat tissue. In this process, the crucial factor is to achieve efficient tissue digestion to extract the desired cells, while ensuring thorough decontamination to enable the proliferation of cells. It is essential for these cells to maintain their functional capacity even after undergoing numerous population doublings in a completely food-safe manner (Fig. 1A). From tissue isolation/biopsy, to the transportation of meat tissue from the slaughterhouse, there is a high risk of environmental contamination, the conditions of the rooms differing greatly from the sterile cell-culture room conditions. The tissues must therefore first be decontaminated before dissection and digestion for cells isolation. We decided to use Perasan MP-2 reagent, which is an FDA-approved antimicrobial for use in water that directly contacts meat, poultry, seafood, plus further processed fruits and vegetables. It contains 15% peracetic acid, 6% hydrogen peroxide, and acetic acid and is an organic oxidizer commonly used in the food industry as there are no environmental risk [19] (Fig. 1B). The mechanisms of action of Perasan are linked to acidification and oxidation effects which can disrupt the cell membrane's permeability and alter protein synthesis, leading to an indirect antimicrobial action [20]. Following PBS washing, the next single most important procedure for preventing infection of cell cultures is careful attention to sterile technique. This initial decontamination of the tissue, followed by the use of sterile materials and reagents in cell isolation and subsequent culture, enables the removal of antibiotics during the ensuing steps of tissue digestion and cell expansion to maintain process using fully edible components. Concerning the tissue digestion to isolate the cells, commonly used collagenase was not included in the food-grade reagents. Another dissociating enzyme, trypsin, was found to be a good replacement as it is edible and easily available. Trypsin is a serine protease produced and secreted as inactive trypsinogen in the pancreas. It has a high specificity for cleaving peptide bonds at the carboxyl side of the basic amino acids arginine and lysine [21]. As trypsin is not specific to ECM digestion, the digestion is generally less effective than using collagenase, but still allows for cell collection after expansion. Here, we tested different dissociating enzymes (collagenase, trypsin and papain, see Supplementary Figure 1) as well as mechanical dissociation or direct seeding of minced tissue to compare the cell isolation efficiency. From the cell expansion found after 4 and 7 days (Supplementary Figure 2), it appeared that both papain and trypsin allowed a suitable cell isolation from bovine adipose tissue, thus trypsin 0.1% was used for the subsequent experiments as it is edible and widely available in biology laboratories. For the cell culture occurring from this step, we used the edible cell culture medium I-MEM (IntegriCulture Minimum Essential Media). This medium is exclusively composed of ingredients approved for food use and specifically designed and developed for the production of cultivated meat [22]. The bovine serum used for complementing the culture medium was also isolated from edible grade bovine plasma (see **4. Materials and Methods**). To assess the efficacy of this edible cell culture medium, in comparison with the standard DMEM with 10% FBS, we conducted evaluations to determine if the cells exhibited adequate proliferative capacity for scaling up from primary isolation to commercial production. We monitored the cumulative population doubling throughout the passages and culture duration (Fig. 1C). No significant difference was found between the use of classic DMEM for the cell culture medium and IMEM. Both media allowed the constant proliferation of the bovine ADSC for at least 35 passages. After 30 days, corresponding to around 10 passages of the cells, the doubling time started to increase in both medium conditions (from 22 $\pm$ 4 h to 46 $\pm$ 15

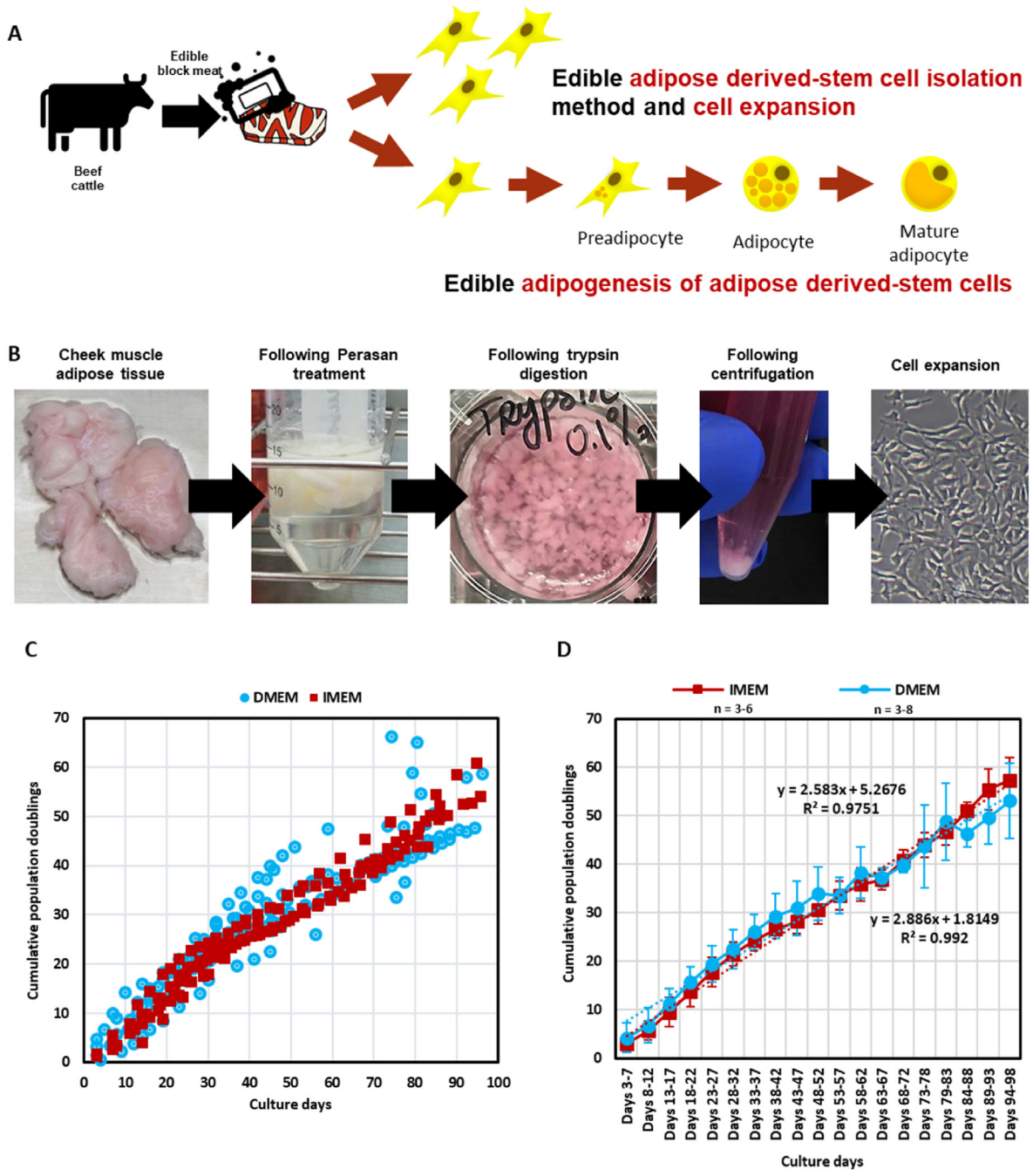


Fig. 1. Edible bovine ADSC isolation and proliferation assessment. A) Fat from cheek muscle tissues was provided from bovine donors at the slaughterhouse, ensuring an edible tissue source for cells isolation. Following transportation and tissue digestion, edible cell proliferation and adipogenesis proliferation of the bovine ADSC was performed. B) Edible cell isolation was performed from disinfected bovine fat tissue using Perasan reagent, followed by trypsin treatment and centrifugation to obtain bovine ADSC. C-D) Bovine ADSC continuous proliferation was assessed in either non-edible DMEM or edible IMEM culture media. The cell number was counted every 2–3 days to plot the cumulative population doubling according to the culture days. The DMEM condition was reproduced on tissues from n = 3–8 independent donors and IMEM from n = 3–6 independent bovine donors.

h at passage 4 and passage 11 respectively for DMEM and from 30 ± 4 h to 38 ± 11 h for IMEM). As the period between each passage was not exactly constant (between 2 and 4 days), an average of the data every 4 days was calculated (Fig. 1D), allowing us to easily monitor the proliferation ability throughout the culture time. Again, no difference was found between the two media conditions, with a proportional cumulative population doubling observed. Starting from the first passage of the isolated bovine ADSC, the cell count could be increased by a factor 53 ± 8 in the DMEM medium condition and 57 ± 5 times in the IMEM medium condition. This implies that from approximately 2×10^5 cells typically obtained at the first passage from around 10 g of fat in muscle tissue, we could obtain a minimum of 1.8×10^{21} in DMEL and 2.9×10^{22} cells in IMEM after around 100 days of serial passages. These findings are particularly significant as they underscore the potential of primary cells to yield a substantial cell content for scaling up cultured meat production.

2.2. Adipogenesis assessment

Our previous study revealed that for inducing adipogenesis in bovine ADSC, the direct addition of more natural fatty acids resulted in higher

lipid storage compared to the conventional insulin-based cocktail used for murine or human stem cells [16]. This distinction is important due to the differences in lipid metabolism between ruminant and non-ruminant mammalian species. While rodents and humans primarily synthesize lipids for storage from glucose, ruminant animals utilize acetate as the principal precursor for lipid generation [23]. As a result, bovine ADSC are generally less responsive to the insulin pathway as glucose is not their major primary source of fatty acid synthesis. Instead, fatty acids can directly activate PPARγ and lipid storage pathways [16], making them more suitable for the food-compatible process of producing cultivated adipocytes.

Although 3D tissue engineering typically yields higher adipogenesis induction due to a more physiological cell organization with cell-cell and cell-material contact [24], it is also crucial to mimic the appearance, structure, and texture of animal-based meats. In this regards, a lipogenesis assessment was performed using collagen-based 3D drop tissues in a 96-well plate, which has been identified as a suitable model for inducing lipogenesis in stem cells [16]. After 2 days of cell proliferation, enabling cell-cell contact, adipogenesis was induced by the addition of 7 fatty acids in the culture medium (phytanic, pristanic, oleic, palmitoleic, myristoleic, erucic and elaidic acids), tested at 3 different concentrations for all fatty acids: 50, 100 and 150 μM, and the lipid storage was measured at days 3, 7 and 14 of differentiation (Fig. 2A–B). The results showed that 100 μM of the 7 fatty acids enabled the most prolific adipogenesis at every time assessment compared to the control without differentiation. This fatty acid concentration allowed a lipid storage 1.4 ± 0.1 fold higher than the control. In contrast, 150 μM was found to be too high, with cells beginning to display a smaller rounder shape which might be linked to cytotoxicity, in comparison to the 100 μM condition where the differentiating adipocytes showed an enlargement of their lipid vesicle size with lower vesicle number due to their progressive fusion (Fig. 2C).

From the 7 fatty acids used, the erucic acid, a fatty acid generally found in plants like wallflower seed, can be associated with the possible occurrence of myocardial lipidosis after consumption [25]. To ensure the production of a safe cultured meat, it was finally decided to remove this fatty acid and to perform the adipogenesis with only 6 fatty acids: phytanic acid, pristanic acid, oleic acid, palmitoleic acid, myristoleic acid and elaidic acid. Edible medium IMEM was also used for the cell differentiation and the maximum passage of the bovine ADSC to be used for adipogenesis induction was assessed for the scale-up application of the cultured adipocytes (Fig. 2D). No significant difference was found between the IMEM and DMEM culture medium conditions, enabling the food-grade production of meat fat *in vitro*. Furthermore, removing erucic acid appeared to have no effect on the adipogenesis induction, displaying up to 2.5 ± 0.1 and 2.9 ± 0.1 fold more lipid storage in DMEM and IMEM respectively, compared to the control condition at passage 7. Up to passage 12, primary bovine ADSC could maintain their differentiation ability with 1.8 ± 0.7 and 1.9 ± 1.1 fold more lipid storage in DMEM and IMEM respectively, compared to the control, suitable to the scale-up application of the cultivated bovine fat production. After this passage, their adipogenesis ability decreases up to showing 1.2 ± 1.1 times more lipid storage at both 15 and 18 passages in IMEM edible medium. By using the expansion assessment, we can thus say that at passage 12, the number of cultured differentiated adipocytes that can be achieved is at least in the region of 2.1×10^{13} cells (corresponding to 26.7 ± 2 fold doubling population from 2×10^{5} cells isolated) in a food-grade condition.

2.3. Lipid composition analysis

The texture, flavor, and caloric value of meat are primarily determined by the protein and fat content present in the muscle, connective and adipose tissues. Fat content, in particular, exhibits significant variation in meat products, with typical cuts of beef ranging from 2.0 to 12.7% [26]. Specialized breeds like Japanese Wagyu beef can even surpass 50% fat content (known as marbling) [5,27]. This abundant fat content greatly contributes to the flavor, nutritional value, and culinary versatility of these products [27,28]. Since the desired fat percentage can vary among different meat products, the ability to produce cultured meat with a controllable fat content is crucial.

The strong association between intramuscular fat content and beef quality, as well as consumer preferences [5], played an important role in our model. We specifically chose to concentrate on recreating the highly marbled Japanese Wagyu beef, known for its superior quality fat composition [27–29]. In this context, the *in vitro* cultivation of fat tissue holds significant value as it offers a tighter control over fat composition and enables a more efficient system for exploring and optimizing the ultimate meat fat qualities. This, in turn, will elicit sensory responses from customers, engaging their olfactory and somatosensory systems.

The composition of fatty acid plays a critical role in determining both the firmness and oiliness of adipose tissue, as well as the oxidative stability of the resulting muscle tissues, all of which significantly impact the flavor and color of the meat product. Specifically, the nutritional value of fats depends essentially on the relative proportions of saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA).

Thus, from 0.5 g of cultured bovine fat, lipid analysis was performed by gas chromatography–flame ionization detection (GC–FID) to monitor in detail the fatty acid composition and the ratios of SFA, MUFA and PUFA from the determination of their chain-length, hydroxylation and unsaturation (Fig. 3A–B). First, it was identified that the final fatty acid composition can be well controlled depending on the cell culture condition and in particular on the fatty acids added to the culture medium during adipogenesis. Three conditions were compared during 14 days of differentiation: using the 7 fatty acids for the adipogenesis of the bovine ADSC, removing erucic acid (6 fatty acids) or using only oleic acid. This last condition was chosen to ensure a fully edible differentiation protocol following Japanese requirements, since the other fatty acids used for the adipogenesis do not appear in the list of the food-grade additives allowed by the Japan Food Chemical Research Foundation [30], even if they are plant-based or animal-based fats usually found in consumer food products. Two control samples were used for comparison: real bovine fat tissues from cheek intramuscular fat and from suet, the latter being the hard fat found around the loins and kidneys. Fatty acids were then classified according to their carbon chain length and carbon-carbon double bonds location (unsaturation).

The predominant SFA in red meats are generally C14:0 (myristic acid), C16:0 (palmitic acid) and C18:0 (stearic acid) [31]. These were found here in both control samples and cultured fat samples. Stearic acid, a cholesterol neutral fatty acid, is beneficial for essential fat ingestion, stimulating fatty acid beta-oxidation and reducing both cardiac and cancer risk in humans [31]. It was found at the highest level in the oleic acid only condition for the cultured fat (26.2%).

For the MUFA and PUFA, the ratio of cis and trans isomers was of importance and was particularly examined. Trans isomers of unsaturated fatty acids for instance, when consumed in excessively large amounts, have been linked to elevated plasma LDL (low-density lipoprotein) cholesterol (the “bad” cholesterol) and reduced HDL (high-density lipoprotein) cholesterol (the “good” cholesterol) state, which is associated with coronary heart disease, cardiovascular disease and type II diabetes mellitus. In contrast, cis fatty acids are beneficial due to their antioxidant and anti-carcinogenic properties [31]. Looking at the isomers of the C18:1 (oleic and elaidic acids), the cis oleic acid (C18:1 n-9), formed from stearic acid (C18:0) by stearoyl CoA desaturase, is a major component of neutral lipids, specifically in Wagyu meat. This trans isomer, elaidic acid, was found at a high percentage in the two conditions using 6 and 7 fatty acids for the differentiation of the cultured fat (25.8 and 16% respectively), compared to the two control samples and the oleic acid only condition, and should be avoided for customer consumption since it can increase the risk of heart and blood vessel disease. The other trans isomers of MUFA and PUFA were found in very limited percentages.

Considering these factors, the cultured bovine fat, differentiated

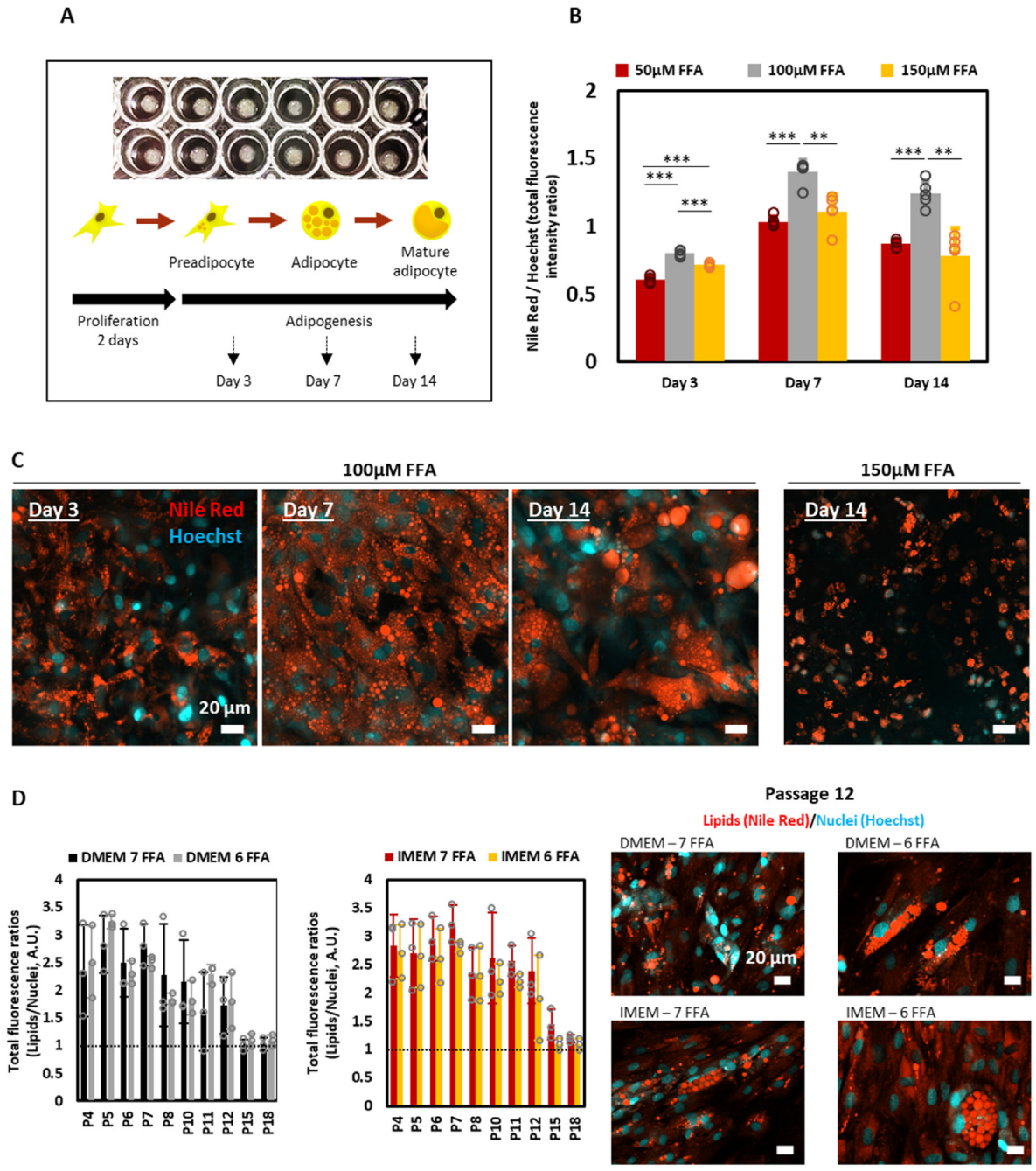
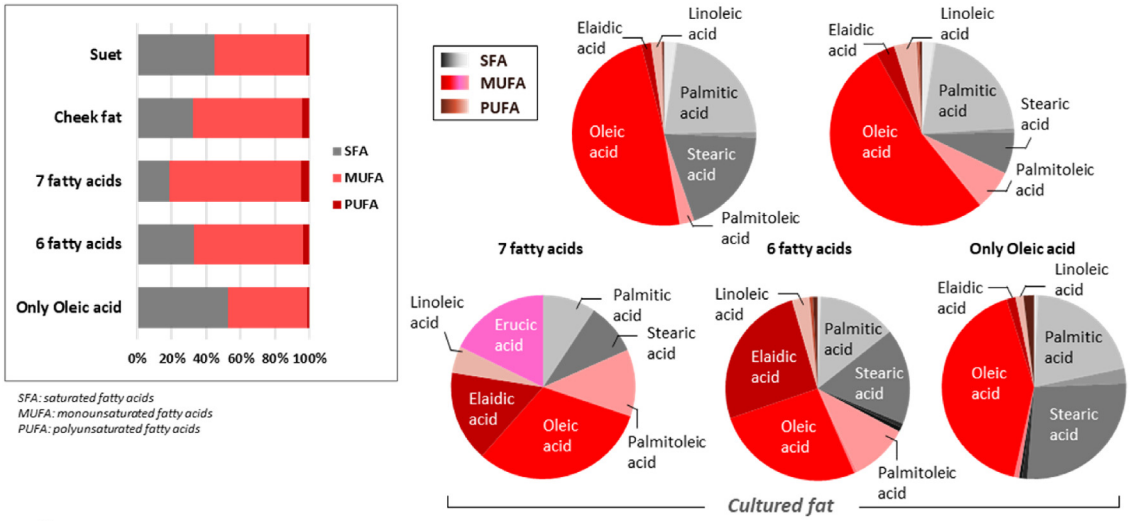


Fig. 2. Edible bovine ADSC lipogenesis assessment. A) Summarized timing of the experiment. Bovine ADSC were initially seeded in 3D drop tissues, allowing for cell proliferation over a period of days. Subsequently, adipogenesis was induced, and samples were examined and quantified at 3, 7, and 14 days post-differentiation. B–C) Nile Red lipid staining and DNA Hoechst staining were performed on the tissues and the total fluorescence intensity ratio of lipids/DNA was measured comparing 3 different concentrations of the added free fatty acids (FFA). The analysis was performed on n = 3 independent donors for cells. Representative images show differentiated bovine adipocytes after 3,7 and 14 days. D) Non-edible DMEM and edible IMEM culture media were compared using either 7 or 6 free fatty acids (FFA) for the adipogenesis induction, measuring the ratio of lipids/DNA fluorescence intensity after 7 days of differentiation. The analysis was performed on n = 3 independent donors for cells. Representative images show differentiated bovine adipocytes at passage 12 after 7 days. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

solely with oleic acid, exhibited a fatty acid composition that closely resembled that of the cheek fat and suet controls. These results differed markedly from the undifferentiated condition, where palmitic acid and stearic acid had the highest ratio content, corresponding to the major components of the sphingomyelin of the cellular membranes [32] (Supplementary Figure 3 and 4). Also, the comparison between 7 and 14 days of culture, as well as between the 7 and 6 fatty acid conditions for the cell culture medium, clearly showed the possibility of directly controlling the fatty acid composition of the differentiating bovine adipocytes since the erucic acid content totally disappeared in the 6 fatty acids condition, becoming similar to the real fat controls. The oleic acid only condition for the adipogenesis also particularly emphasized the transformation of the added cis oleic acid by the differentiating adipocytes during the lipid storage, enabling a final composition of various fatty acid components and was surprisingly found to be a better fit to the real fat controls.

This lipid composition, which closely mimics the authentic fat content of the meat, plays a crucial role in achieving a high intramuscular marbled fat content. This characteristic elicits a tender and juicy



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		Omega Nomenclature	Delta Nomenclature	Isomer	Systematic Name (IUPAC name)	Trivial name	Real meat fat: Suet	Real meat fat: Cheek fat	Cultured fat: 7 fatty acids	Cultured fat: 6 fatty acids	Cultured fat: Only Oleic acid
C8					octanoic acid	caprylic acid	0	0	0	0	0
C10					decanoic acid	capric acid	0	0	0	0	0
C12					dodecanoic acid	lauric acid	0	0	0	0	0
C14					tetradecanoic acid	myristic acid	2.0	2.0	0	0.3	0.3
C15					pentadecanoic acid	pentadecylic acid	0.3	0.4	0	0.3	0.4
C16					hexadecanoic acid	palmitic acid	22.4	21.7	9.3	13.8	20.5
C17					heptadecanoic acid	margaric acid	1.0	0.7	0	0	2.6
C18					octadecanoic acid	stearic acid	18.9	7.3	9.1	16.9	26.2
C20					icosanoic acid	arachidic acid	0.1	0	0	0	0
C22					docosanoic acid	behenic acid	0	0	0	0.7	0.9
C24					tetracosanoic acid	lignoceric acid	0	0	0	0.8	0.5
C16:1		n-7	Δ9	cis	(9Z)-hexadec-9-enoic acid	palmitoleic acid	2.5	6.9	11.8	10.3	0.04
		n-7	Δ9	trans	(9E)-hexadec-9-enoic acid	Palmitelaidic acid	0.1	0.2	0	0.5	0.84
C17:1		n-8	Δ9	cis	(9Z)-heptadec-9-enoic acid	margaroleic acid	0	0	0	0	0
C18:1		n-9	Δ9	cis	(9Z)-octadec-9-enoic acid	oleic acid	48.7	52.7	31.4	26.4	40.7
		n-9	Δ9	trans	(9E)-octadec-9-enoic acid	elaidic acid	1.7	3.3	16	25.8	1.54
C18:2		n-6	Δ9,12	cis	(9Z,12Z)-octadeca-9,12-dienoic acid	linoleic acid	1.9	4.0	4.7	3.1	1.37
		n-6	Δ9,12	trans	(9E,12E)-octadeca-9,12-dienoic acid	linoelaidic acid	0.0	0.0	0	0	0
C18:2		n-6	Δ9,12	trans	(9Z,12E)-octadeca-9,12-dienoic acid	cis-9-trans-12-linoleic acid	0.0	0.2	0	0	0
		n-6	Δ9,12	trans	(9E,12Z)-octadeca-9,12-dienoic acid	trans-9-cis-12-linoleic acid	0.0	0.1	0	0	0
C18:3		n-3	Δ9,12,15	cis	(9Z,12Z,15Z)-octadeca-9,12,15-trienoic acid	α-linolenic acid	0.1	0.2	0	0.6	0
		n-6	Δ6,9,12	cis	(6Z,9Z,12Z)-octadeca-6,9,12-trienoic acid	γ-linolenic acid	Not analyzed	Not analyzed	Not analyzed	Not analyzed	0
C20:1		n-9	Δ11	trans	(11E)-icos-11-enoic acid	11-trans-eicosenoic acid	0	0	0	0	0
C22:1		n-9	Δ13	cis	(13Z)-docos-13-enoic acid	erucic acid	0	0	17.8	0	0
		n-9	Δ13	trans	(13E)-docos-13-enoic acid	brassidic acid	0	0	0	0	0
						others	0	0	0	0	2.3*
						Total	100	100	100	100	100

*C15:1 (10Z-pentadecenoic acid) = 1.1 %, C20:3 (11Z,14Z,17Z-Eicosatrienoic acid) = 0.6 %, C20:4 (Arachidonic acid) = 0.4 %, and C24:1 (15Z-tetracosenoic acid) = 0.2 %.

Fig. 3. Fatty acid composition of the bovine cultured adipose tissue. A-B) Lipid analysis was performed by gas chromatography–flame ionization detection (GC–FID) on 0.5 g of cultured bovine adipose tissue after 14 days of adipogenesis. Fatty acid composition by determining their chain-length, hydroxylation and unsaturation were compared in terms of ratios, compared to 2 control samples of real bovine fat: bovine suet (commercially available, unrefined suet derived from Japanese brown cattle) and cheek intramuscular fat (crossbreed of beef cattle and dairy cattle, female, 30 months old). A) Comparison in term of saturation types of fatty acids depending of the culture condition. B–C) Detailed comparison of fatty acids for each culture condition. Each condition was performed with tissue or cells obtained from one donor. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

sensation in the meat, accompanied by an enhanced overall flavor due to the production of nonvolatile breakdown products and volatile aromatic compounds when the beef is cooked [33]. The exact relationship between fatty acids, their derivative volatiles and the flavor profile of meats remains immensely complex. Some trends were observed however, such as the positice association between palmitic, palmitoleic, and oleic acid content with favorable palatability. Particularly, in high quality beef meat, such as Wagyu meat, the presence of high levels of oleic acid is vital for tenderness and juiciness [34]. In contrast, a high content of polyunsaturated fatty acids is associated with undesirable flavors [35]. These findings substantiate the high-quality composition of the cultured bovine adipose tissue across various aspects.

3. Conclusion

The findings presented in this study offer valuable insights into the application of cell-cultured meat and can guide ongoing research in developing products that align with consumer preferences by adjusting their sensory properties. The observed proliferation rate and the adipogenesis differentiation of the isolated primary bovine ADSC provide realistic foundations for scaling up the bio-manufacture of edible meat alternatives. In this model, the final lipid composition could be effectively controlled by adjusting the fatty acids introduced during the differentiation process, resulting in organoleptic characteristics that closely resemble the desired composition of real fat. Notably, a relatively high ratio of oleic acid was observed, which holds particular significance for the production of high-grade Japanese Wagyu meat. However, while this point is highly encouraging, it still needs to be thoroughly assessed, particularly through real organoleptic studies involving willing participants, to determine more definitively the extent to which sensory evaluation and the final taste closely resemble that of meat fat.

This *in vitro* bovine adipose tissue cultivation method can be an asset for optimizing the taste, nutrition, mouthfeel, juiciness, and other qualities relevant to human health and consumer perception not only for cultured meat production but also in other processed foods and various applications. For instance, the use of cultured fat cells along with plant-based protein can markedly improve the quality and consumer perception of plant-based meats without compromising their sustainability, making cultured adipocytes a key component for both plant-based and animal-based alternative meats. Another possibility is also to use cell-based alternative meat to enhance the nutritional value of cultured meat, by the incorporation of additional vitamins for instance. However, our study has focused on faithfully recreating the composition and characteristics of real meat to ensure consumer acceptance and perception of this novel food source.

One of the concerns addressed in this study was related to the use of primary cells which are generally not suitable for longer term culture required in the production of meat products solely through cell culture and tissue engineering. In response to this limitation, various start-up companies worldwide have opted to develop immortal bovine cell line instead to overcome this limitation. However, our findings indicate that this is not necessary as regular isolations can be performed to obtain fresh cells, along with validation procedures to ensure the consistency. These bovine ADSC demonstrated their ability to efficiently and safely differentiate into adipose or adipose-like tissue while maintaining their capacity even after multiple population doublings. The subsequent phase will involve transitioning to higher-density bioreactor systems to facilitate the expansion of cell production on an industrial scale. Moreover, the entire process will need to be adaptable to completely non-animal derived components in the culture media, while also ensuring low-cost culture media without compromising performance even, particularly when upscaling to bioreactor-based culture systems.

4. Materials and methods

4.1. Materials

Collagen type I solution from porcine skin in 5 mM acetic acid (892,171) was purchased from Nippi. NaOH solution was sourced from Wako Chemicals. Hexane (17,922–94), methanol (21,915–35), NaOH (31,511–05), NaCl2 (31,320–34) for fatty acid analysis and PBS (07269–84) were sourced from Nacalai Tesque. Bovine fibrinogen (F8630), bovine thrombin (T4648) and BF3/MeOH/MeOH solution (B1252) were purchased from Sigma Aldrich. CaCl2 (58,001–17) was purchased from Kanto Chemical. FAME Mix was purchased from SUPELCO. Cell culture treated 96-well plates (3860–096) were sourced from Iwaki. Perasan MP2-J was sourced from EnviroTech.

4.2. Livestock tissues used and bovine blood serum preparation

The bovine muscle adipose tissues in this study were derived from Japanese black cattle (female, 27–30 months old), kindly sent by the University of Tokyo (Department of Mechano-Informatics, Graduate School of Information Science and Technology). Cheek muscle adipose tissues were obtained from commercial fresh beef isolated after slaughter (Tokyo Shibaura Zouki) on the day of slaughter and sent to the laboratory packed in ice. The edible bovine blood serum was prepared by modifying the edible grade bovine plasma derived from Proliant (New Zealand). $CaCl_2$ was added to the bovine plasma at a final concentration of 20 mM and incubated to solidify overnight at 37 °C, then filtered through a 0.22 μm strainer. The obtained liquid was used as the bovine blood serum. Due to the inherent complexity of bovine serum composition, which can vary among different donors, it can have a potential impact on cellular behavior. For the scale-up of the process it will be necessary to ensure the homogeneity by mixing serum from several donors, which was not the case in the current study.

Ethical approval was not required for this study since the bovine ADSC were isolated from commercial fresh beef obtained after slaughter and sent to the laboratory.

4.3. Bovine ADSC isolation

The received cheek muscle bovine fat tissue was first washed in 0.2% Perasan (the concentration was chosen as the recommended maximum dose for meat application purposes by the manufacturer as approved by the FDA) prepared in PBS for 5 min at room temperature, followed by three times PBS washing for 5 min each time. This step is probably cytotoxic for the bovine cells on the surface of the meat but is necessary to ensure the decontamination of the tissue before its digestion to isolate the viable cells inside. The visible blood vessels were then removed and the bovine fat tissue was minced using sterilized tweezers and scissors until final tissue pieces of around 3 mm size were obtained. The minced tissues were moved to a 6-well plate (around 2 g of tissue/well to digest) and 3 mL of trypsin 0.1% was added per well with incubation in a plate shaker for 30 min at 280 rpm and 37 °C. Following incubation, 5 mL of IMEM containing 10% of bovine blood serum was added to each well to stop the trypsin reaction and pipetting was performed to complete the breakdown of the digested tissue. The solution was finally centrifuged at 1000 rpm for 10 min at room temperature, the pellet was filtered through a 40 μm cell strainer and cells were cultured in IMEM with 10% of bovine blood serum. After 3 days of attachment and expansion, the cells were washed 3 times with PBS to remove all the remaining tissue debris and the medium was renewed every 2–3 days until 70% of confluence where the cells were passaged. After one passage, the remaining adherent cells were considered to be ADSC and were expanded.

4.4. CMF preparation

The collagen type I solution was first neutralized by mixing gently on ice with a buffer solution containing 10X PBS and 0.05 N NaOH at a ratio of 4:1 of collagen:neutralizing buffer. The neutralized collagen solution was then incubated for 30 min in a water bath at 37 °C until it became a gel before being freeze-dried for 48 h (FDU-2200, EYELA). Following freeze-drying, the lyophilized collagen sponge was then crushed into small pieces using tweezers and homogenized on ice in 85% ethanol for 6 min at 30,000 rpm, before being centrifuged at 10,000 rpm for 3 min at room temperature. The supernatant was discarded and the pellet was homogenized again on ice in 70% ethanol followed by centrifugation at 10,000 rpm for 3 min at room temperature. The pellet was then washed twice in MilliQ water, and the collagen fibers in suspension were ultrasonicated (Ultrasonic processor VC50, SONICS) 10 times for 20 s with a 10 s cooling time between each, on ice. A last freeze-drying step was performed for 48 h on the collagen fibers suspension and the collagen microfibers (CMF) obtained were stored in a desiccator until use.

4.5. 3D gel embedded culture

To construct the adipose tissues by 3D culture, CMF were first weighed and washed in sterile MilliQ water by being centrifuged for 1 min at 16,083 g to obtain a final concentration in the tissues of 1.2 wt%. The bovine ADSC were added after trypsin detachment and centrifuged for 1 min at 1970 g to obtain a final cell concentration of 5×10^6 cells/mL. The pellet containing CMF and ADSC was then mixed in a fibrinogen solution at 6 mg/mL final concentration and thrombin solution was added to obtain a final concentration of 3 U/mL. Finally, 2 μL drop tissues were seeded in a 96-well plate and gelated for 15 min in an incubator at 37 °C. Then 300 μL of medium (IMEM with 10% bovine blood serum) was added to the drop tissues. For adipogenic differentiation, 2 days of proliferation were first necessary to allow the ADSC proliferation until reaching a suitable cell-cell interaction required for the adipogenesis [36]. The medium was then switched for IMEM with 10% bovine blood serum containing different free fatty acids [15]: Pristanic [15]: pristanic acid (Funakoshi, 11–1500), phytanic acid (Sigma Aldrich, P4060), erucic acid (45629-F), elaidic acid (Sigma Aldrich, 45,089), oleic acid (Sigma Aldrich, O1383), palmitoleic acid (Sigma Aldrich, 76,169), myristoleic acid (Sigma Aldrich, 41,788). The 300 μL of differentiation medium were then renewed every 2–3 days.

4.6. Lipogenesis analysis

Following adipogenesis, the cells were fixed in 4% paraformaldehyde at 4 °C overnight and 100 ng/mL Nile Red (TCI, N0659) was applied for lipid staining at room temperature for 30 min, with 300 nM DAPI (Invitrogen, D21490) counterstaining. For the calculation of lipid production from ADSC, the 3D tissues' Z-stack images were taken with the same exposure time, brightness, and contrast for all conditions. Then the Maximum Intensity Projection (MIP) of Z-slice's images in Nile Red and Hoechst of each 3D tissue was performed. For this, a Confocal Quantitative Image Cytometer CQ1 (Yokogawa, Tokyo, Japan) was used, with the following channels: Nile Red using the 561 nm laser (power 20%), Ex: 617–673 nm, exposure time 500 ms, and Hoechst using the 405 nm laser (power 100%), Ex: 447–460 nm, exposure time 500 ms.

The total fluorescence intensity was finally calculated from the total Nile Red's intensity of the MIP images divided by the total Hoechst intensity in each 3D tissue using ImageJ software (version 1.53j, NIH, USA).

4.7. Analysis of lipid composition

Analysis of lipid composition was performed at NISSIN FOODS Holdings, Global Food Safety Institute. Lipids were extracted from 0.5 g of the cultured bovine fat or the real bovine fat with hexane. The extracted lipids were collected in a test tube and hexane was removed by blowing nitrogen gas. Then, 1.5 mL of 0.5 M sodium hydroxide in methanol was added, and the lipids were saponified by reacting in a boiling water bath for 7 min. After cooling at room temperature, 2 mL of 14% boron trifluoride/MeOH/MeOH was added and reacted in a boiling water bath under nitrogen gas for 5 min to methylate the samples. After cooling at room temperature again, 2 mL of hexane and 5 mL of saturated solution of sodium chloride were added and the mixture was shaken, and phases were separated and then washed with 2 mL of hexane. The hexane phase was separated and transferred into a 5 mL volumetric flask and replenished by hexane and subjected to GC analysis. The two real bovine fat controls used for comparison in the analysis came from bovine suet (commercially available, unrefined suet derived from Japanese brown cattle) and cheek intramuscular fat (crossbreed of beef cattle and dairy cattle, female, 30 months old). Suet control is a beef-derived fat commonly used in various dishes and was chosen for its aromatics generally associated with beef roasted taste as well as cooked animal fat taste [37].

4.8. Chromatographic analysis

Composition of fatty acid analysis was conducted using a Shimadzu GC-2010 plus gas chromatograph (Shimadzu Corporation, Tokyo, Japan) with a flame ionization detector (FID) and capillary column DB-23 Agilent Technologies (60 m × 0.25 mm) with a stationary phase (50% cyanopropyl, methyl-polysiloxan) with the thickness of 0.15 μm. The injection volume was 1.0 μL, the inlet temperature was 250 °C, the split ratio was 1:50, and hydrogen was used as the carrier gas. The temperature program was 50 °C for 1 min, then the temperature was increased to 175 °C at 25 °C/min, then increased to 230 °C at 25 °C/min, held for 5 min, and cooled at 5 °C/min. The identification of fatty acids was performed by comparing their retention times with those of reference standards (fatty acid methyl ester (FAME) Mix, included 37 FAMEs).

4.9. Statistical analysis

Statistical analysis was performed using EzAnova (version 0.98, University of South Carolina, Columbia, SC, USA) software. The detail of the number of n corresponding to the number of independent experiments using isolated bovine primary cells from different bovine donors, or independent samples, is displayed in the captions. A two-way ANOVA was applied with time set as “paired or repeated measures” and the treatment as classic analysis or “unpaired” which led to a pairwise comparison, with the Tukey's HSD post hoc test for the multiple comparisons. Error bars represent SD. p values < 0.05 were considered to be statistically significant.

CRediT authorship contribution statement

Fiona Louis: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Mai Furuhashi:** Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. **Haruka Yoshinuma:** Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. **Shoji Takeuchi:** Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing. **Michiya Matsusaki:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The authors declare no competing financial interests but the following

competing non-financial interests concern the authors Mai Furuhashi and Haruka Yoshinuma who are employees of the company Nissin Foods Holdings Co., Ltd., where the fatty acids composition analysis was performed.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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