Long-term administration of whey alters atrophy, gene expression profiles and dysfunction of salivary glands in elderly rats

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Abstract

Salivary glands in elderly individuals commonly exhibit morphological changes and dysfunction resulting in xerostomia. Long-term (4-week) drinking of whey prevented and/or restored age-dependent decline of salivary volume and protein concentration, and atrophy of sublingual glands (SLGs) significantly in 88-week-old rats. The transcripts of 42 genes were up-regulated and 7 genes were down-regulated by more than 1.5-fold change with FDR $\leqq$ 0.1 after whey-drinking. The expression levels of genes associated with salivary proteins and tissue repair were significantly increased, while those associated with lipid metabolism were decreased. Venn diagram analysis revealed that expressions of 13 genes, including *Tcfap2b* and *Abpa*, were induced significantly by whey-drinking. Furthermore, secretory protein levels in SLGs and saliva were revealed by immunoblot analysis. This is the first study to report that whey-administration can prevent and/or restore age-dependent atrophy and functional decline of SLGs in relation to gene expression and thus may serve as a functional food ingredient.

Keywords:

Whey, Aging, DNA microarray, Salivary glands, Atrophy, Dysfunction

Abbreviations: AR, androgen receptor; AP-2, activating protein-2; FDR, false discovery rate; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SLGs, sublingual glands; OC, water-drinking elderly rat; OJ, whey-drinking elderly rat; PRP, proline-rich protein; qRT-PCR, quantitative reverse transcription-polymerase chain reaction

1. Introduction

Xerostomia, which is also referred to as dry mouth, is a common problem among the elderly (Gonsalves *et al.*, 2008; Sun *et al.*, 2013). Xerostomia leads to infectious conditions, such as periodontal disease and caries, in the oral cavity (Sreebny, 2000; Guggenheimer & Moore, 2003); these, in turn, lead to an increased risk of atherosclerosis, cardiovascular disease (Meurman *et al.*, 2004) and diabetes (Darre *et al.*, 2008). Thus, xerostomia can result in not only oral diseases, but also systemic diseases. In addition, xerostomia causes dysphagia (Guggenheimer & Moore, 2003), resulting in a reduced quality of life, and increases susceptibility to aspiration pneumonia (Kikutani *et al.*, 2015), which can cause mortality.

Salivary glands consist of three major glands, i.e., the parotid glands, submandibular glands and sublingual glands (SLGs), as well as numerous minor glands. In the unstimulated condition, the parotid glands have a very low secretion rate. In contrast, the submandibular glands and SLGs secrete relatively more saliva under unstimulated conditions (Proctor & Carpenter, 2007). The glands are innervated by autonomic nerves (Proctor & Carpenter, 2007). Noradrenaline released from sympathetic nerves stimulates salivary secretion through α_1- and β-adrenoceptors. Activation of α_1-adrenoceptors induces fluid secretion from the salivary glands via an increase in the intracellular concentration of calcium, while activation of β-adrenoceptors induces protein secretion from the glands via the activation of protein kinase A. Acetylcholine released from parasympathetic nerves interacts with

the M_1- and M_3-muscarinic cholinergic receptors to induce fluid secretion from the salivary glands via activation of the calcium signaling pathway (Proctor & Carpenter, 2007). An age-related decrease in salivary secretion has been recognized during both the unstimulated condition (Ship *et al.*, 2002; Pan *et al.*, 2009) and the stimulated condition (Inoue *et al.*, 2003; Choi *et al.*, 2013).

Whey, a co-product of cheese manufacturing, can supply not only nourishment, but also many biologically active components to humans. For example, whey protein stimulates protein synthesis in relation to gene expression in skeletal muscle after exercise (Kanda *et al.*, 2013; Kanda *et al.*, 2014) as well as the accretion of muscle protein in the elderly (Pennings *et al.*, 2011). In addition, it has been shown to reduce tumorigenesis in the rat colon (Xiao *et al.*, 2005). Whey protein also has an insulinotropic effect in both type 1 (Hwang *et al.*, 2012) and type 2 (Pasin & Comerford, 2015) diabetes. Beta-lactoglobulin, a major component of whey, acts as a molecular carrier and alters the bioaccessibility of linoleate/linoleic acid, reducing the risk of cardiovascular disease (Le Maux *et al.*, 2012). Thus, whey is a functional food with possible therapeutic applications.

To the best of our knowledge, no study has assessed the impact of whey on the aging process in the salivary glands; thus, in the present study, we examined the effects of whey supplementation on age-related changes in morphology, gene expression and function of rat salivary glands. We found a new function of whey that recover age-dependent atrophy and functional decline of the SLGs in relation to gene expression.

2. Materials and methods

2.1. Experimental animals and collection of saliva

Eight-week-old male Wistar rats (body weight: 211 ± 18 g) were purchased from SLC, Inc. (Shizuoka, Japan) and given a standard laboratory chow (MF; Oriental Yeast,

Tokyo, Japan). At the age of 12-week (young, body weight: 294 ± 6 g) and 84-week (elderly, body weight: 491 ± 5 g), rats were divided into water-drinking group and whey-drinking group. One young group and one elderly group were provided water ad libitum for 4 weeks and one young group and one elderly group were provided cheese whey from Jersey cattle (donated by Ooyama Ranch Inc., Kagawa, Japan) ad libitum for 4 weeks. Compositional characteristics of whey are in Supplementary table1. They were maintained in a temperature-controlled environment (22 ± 2°C) with a 12-h-light/12-h-dark cycle in accordance with the guidelines established by the Animal Care Committee of Tokushima University Graduate School. Salivary glands were rapidly removed from rats sacrificed by a blow to the head under chloroform-inhalation. Cevimeline (10.0 mg/kg)(Daiichi-Sankyo Pharmaceutical Co. Tokyo, Japan) was injected intraperitoneally into rats. During the first 30 min after cevimeline-injection, saliva was collected by pipette.

2.2. Histological analysis

Salivary glands for light microscopic examination were fixed by immersion in 10% phosphate-buffered formalin and then processed for paraffin sections. Routinely, 5-μm sections were cut and stained with hematoxylin and eosin.

2.3. DNA microarray experiments

Four rats were randomly selected from each of the water-drinking and whey-drinking groups. The total RNA was isolated from the SLGs of the rats using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) and QIAcube (Qiagen GmbH). RNA purity was assessed by the ratio of the absorbance at 260 and 280 nm measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) and

was examined using an Agilent2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). First-strand cDNA was synthesized from 400 ng of total RNA using the Whole Transcript (WT) Expression Kit (Ambion, Austin, TX, USA) according to the supplier's protocols. The resultant cDNA was fragmented and end-labeled with the GeneChip® WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA, USA). Then, 5.5 μg of the fragmented and labeled DNA target was hybridized to the Affymetrix GeneChip® Rat Gene 1.0 ST Array (Affymetrix) at 45°C for 17 h in a GeneChip® Hybridization Oven 640 (Affymetrix) according to the recommended experimental protocols provided by the supplier. This array comprised more than 722,254 unique 25-mer oligonucleotide features constituting more than 27,342 probe sets for known and unknown genes. The hybridized arrays were washed and stained in a GeneChip® Fluidics Station 450 and scanned with a GeneChip® Scanner 3000 7G (Affymetrix), and GEL files were generated for each array. In each comparison experiment, the intensity levels for the two chips were normalized by dye swap and flag treatments using Affymetrix Expression Console Software (Affymetrix).

2.4. Analysis of microarray data

Microarray data were imported into GeneSpring GX version 12 (Agilent, Santa Clara, CA, USA). This software package allows multi-filter comparison using data from different experiments to perform normalization and the generation of restriction lists and functional classifications of the differentially expressed gene. The signal intensities of the selected genes that were up-regulated or down-regulated by at least 1.5-fold when compared to a control group were extracted by this software and a false discovery rate (FDR) value was calculated using the Significant Analysis of Microarrays method (Tusher *et al.*, 2001). FDR value was rounded off a number of the decimal point third position and the expression change was taken as informative when FDR value was $\leqq$ 0.1.

152

153 2.5. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

154 The differential expression of selected genes was assessed by qRT-PCR. cDNA was
155 synthesized from 500 ng of RNA extracted from the SLGs using the High Capacity
156 cDNA Reverse Transcription Kit (Applied Biosystems, Foster, CA, USA). cDNA was
157 amplified using the StepOnePlus™ Real-Time PCR System (Applied Biosystems). The
158 primers listed in Supplementary table 2 were designed using Primer-BLAST software
159 (NCBI). Amplified cDNA was detected using the Power SYBR Green PCR Master Mix
160 Reagent kit (Applied Biosystems). The PCR conditions were as follows: incubation for
161 10 min at 95°C, followed by 40 cycles of denaturation for 10 s at 95°C, annealing for 15 s
162 at 60°C and extension for 15 s at 72°C. The target and reference genes were amplified
163 on the same plate. A non-template control was included for all of the primer pairs in
164 each run. Data were analyzed using StepOnePlus™ software (version 2.3; Applied
165 Biosystems). The expression values were normalized to that of β-actin as an endogenous
166 control as it showed little variation in expression across the sample sets. The mean fold
167 change of expression in the SLGs of whey-drinking rats compared with that of
168 water-drinking rats using the 2-ΔΔCT method. Analyses were performed in triplicate
169 using RNA samples from at least three different rats.

170

171 2.6. Gel electrophoresis and Western blot analysis.

172 SLGs and saliva samples were dissolved with Laemmli sample buffer (Bio-Rad,
173 Hercules, CA, USA) and the samples with 15 μg of protein or 15 μl were subjected to
174 SDS-polyacrylamide gel electrophoresis (PAGE) on 10%, 12.5% and 15% gradient gels.
175 For immunoblot analysis, the separated proteins were transferred onto a nitrocellulose
176 transfer membrane (Hybond ECL; Amersham Biosciences, Little Chalfont, UK).
177 Membranes were blocked overnight with 0.1% Tween 20 in Tris-buffered saline pH 7.4.

178 The blots were then probed with rabbit anti-basic proline-rich protein (PRP, 1:10 000; a
179 kind gift from Prof. David Castle, University of Virginia (Castle & Castle, 1993)), rabbit
180 anti-cystatin S (1:500, Sino Biological Inc., Beijing, China), or rabbit anti-amylase
181 (1:1500, Calbiochem, Darmstadt, Germany) antibody. The membranes were washed
182 with Tris-buffered saline containing 0.1% Tween 20 and incubated with horseradish
183 peroxidase-linked donkey anti-rabbit IgG, whole Ab (1:5000, GE Healthcare,
184 Piscataway, NJ, USA) for 1 h. Immunoblots were processed using enhanced
185 chemiluminescence (ECL) (Amersham) and the signals were visualized on a Chemi Doc
186 apparatus (Bio-Rad, Hercules, CA, USA). The bands were quantified using Quantity
187 One software (Bio-Rad).
188
189 2.6. Statistical analysis
190 Data are expressed as the mean value ± standard error. To test for statistically
191 significant differences between two groups, a paired Student's t-test was used for
192 biochemical tests, qRT-PCR and Western blot analysis. A p value of less than 0.05 was
193 considered to be statistically significant. In microarray analysis, Moderated t-test was
194 used.
195
196 ## 3. Results
197 3.1. Animals
198 Body weight at the beginning and end of the study, drinking volume, salivary gland
199 weight and salivary protein concentration were not significantly different between the
200 water-drinking (control) young rats and the whey-drinking young rats (Table 1). In
201 elderly rats, body weight at the beginning and end of the study, drinking volume,
202 parotid and submandibular gland weight were not also significantly different between
203 the water-drinking group and the whey-drinking group. SGL weight, salivary volume
204 and salivary protein concentration were significantly higher in whey-drinking elderly
205 rats compared to water-drinking elderly rats.

206

207 ## 3.2. Morphology

208 To evaluate the effect of whey-administration on the morphology of the SLGs, histology
209 was performed on SLGs tissue sections using hematoxylin and eosin staining (Fig.
210 1A-D). Atrophy was detected in the SLGs of elderly water-drinking rats (Fig. 1C), but
211 not in the SLGs of the younger water-drinking rats (Fig. 1A). Whey-drinking did not
212 affect the morphology of the SLGs in the younger whey-drinking rats (Fig. 1B), and
213 age-dependent atrophy of the SLGs was not detected in the elderly whey-drinking rats
214 (Fig. 1D). These results suggested that whey-drinking prevented and restored
215 age-dependent atrophy of the SLGs.

216

217 ## 3.3. Microarray

218 Microarray chips are a powerful technology that allows the simultaneous measurement
219 of the expression levels of thousands of genes. The resulting expression profiles have led
220 to dramatic advances in the understanding of cellular processes at the molecular level.
221 Total RNA was isolated from the SLGs of eights rats in the 84-week-old group; samples
222 from the rats in the water-drinking group and the whey-drinking group (n = 4 each)
223 were compared. Of 27,342 genes, 42 genes were up-regulated by at least 1.5-fold change
224 with FDR $\leqq$0.1 in the whey-drinking group when compared to the water-drinking
225 group (Table 2). The up-regulated genes included those encoding major salivary families
226 of specific secretory proteins (Scarano *et al.*, 2010), i.e., PRP genes (*Prp2, Prol1, Prog1,*
227 *Prp15*), cystatin genes (*Cyss, Vegp2*), and amylase gene (*Amy1a*). The expression of
228 tissue-specific genes, i.e., kallikreins (*Klks3, Klk1c10, Klk1*) and kallikrein-related
229 genes (*Ton, Klk1l, Klk1c10l2*), was induced in the whey-drinking group. The levels of
230 androgen-binding protein gene (*Abpa*) and androgen-related protein genes (*Smr3a*)
231 were also increased in the whey-drinking group when compared to the water-drinking
232 group.

233 Of 27,342 genes, 7 genes were down-regulated by at least 1.5-fold change with FDR
234 ≦0.1 in the whey-drinking group when compared to the water-drinking group (Table 3).
235 The down-regulated genes included those associated with lipid metabolism,
236 transcription regulation, cancer and senescence.
237 In order to investigate the common genes showing fluctuating expression levels in
238 the four sets, Venn diagram analysis was performed (Fig. 2 A). Each set comprised
239 microarray data from the whey-drinking group vs. those from the water-drinking group.
240 In the first (Fig. 2 B-a), second (Fig. 2 B-b), third (Fig. 2 B-c) and fourth (Fig. 2 B-d) sets,
241 the whey-drinking group showed 512, 238, 473, and 938 entities with more than a
242 1.5-fold difference when compared to the water-drinking group, respectively. Among the
243 four sets, 21 entities were overlapped (Fig. 2A). Because this array comprised more than
244 722,254 unique 25-mer oligonucleotide features constituting more than 27,342 probe
245 sets, 21 entities represent 13 genes. The 13 genes are listed in Table 4. Transcription
246 factor AP-2 gene (*Tcfap2b*), which is a salivary gland-specific transcription factor gene
247 (Hu & Gallo, 2010), and androgen-binding protein alpha (*Abpa*), which is a pheromone
248 gene (Karn & Laukaitis, 2009), are induced by whey-administration, suggesting that
249 androgen-dependent transcription programs functioned together with a tissue-specific
250 collaborating factor, AP-2, in whey-drinking rats.
251
252 3.4. qRT-PCR
253 In order to verify the results of the microarray analysis, selected genes with an
254 expression level that differed by at least 1.5-fold between the whey-drinking group and
255 the water-drinking group were confirmed using qRT-PCR. Whey administration
256 induced an approximately 15-fold up-regulation of the *Cyss* and *Prb1* genes and an
257 approximately 4-fold up-regulation of the *Tcfap2b* and *Abpa*. In addition, whey
258 administration induced a 2-fold up-regulation of the *Amy1a* and *Lyz2* genes. All of the
259 results of qRT-PCR analysis were consistent with the microarray expression profiles
260 (Fig. 3).

261

262 3.5. Western blotting

263 Western blot analysis was used to quantify the changes in levels of selected proteins in
264 the SLGs (Fig. 4 A) and saliva (Fig. 4 B) in response to whey-drinking. PRP, cystatin S
265 and amylase levels in the SLGs were increased by 3-, 4.5- and 5-fold, respectively, in the
266 whey-drinking group in comparison with the water-drinking group (Fig. 4A). Salivary
267 PRP, cystatin S and amylase levels were increased by 2.5- to 3-fold in the whey-drinking
268 group in comparison with the water-drinking group (Fig. 4B). These results suggested
269 that whey-drinking prevented and/or restored the age-dependent functional decline in
270 SLGs and saliva.

271

272 ## 4. Discussion

273 This is the first study to report that whey prevented and recovered age-dependent
274 atrophy and functional decline of the salivary glands in relation to gene expression.
275 Saliva is a unique body fluid that continually bathes the oral cavity and larynx to aid in
276 essential functions, such as mastication, oral microbial defense, gustation, lubrication,
277 speech, deglutition, digestion, mineralization of teeth, and the protection of mucosal
278 tissues (Humphrey & Williamson, 2001; Amerongen & Veerman, 2002). The
279 fundamental functions of saliva are strictly connected to its composition that consists in
280 a complex hypotonic aqueous solution containing proteins, peptides, enzymes, hormones,
281 sugars, lipids and others (Messana *et al.*, 2008). Recent proteomic approaches have
282 revealed that more than 2,400 proteins are contained in saliva (Castagnola *et al.*, 2011).

283 PRPs account for more than 60% of the weight of the total salivary proteome
284 (Messana *et al.*, 2008). They have been divided into three classes: basic PRP, acidic
285 PRP, and glycosylated PRP. Among the PRP genes induced by whey-drinking, *Prb1*
286 belongs to the basic PRP group, *Prp2* and *Prp15* belong to the acidic PRP group and
287 *Prpg1* belongs to the glycosylated PRP group. According to our results, the levels of
288 PRPs were also increased in the SLGs and saliva by whey-drinking. Packaging of PRPs

289 into the secretory granules of salivary glands may be important in the reduction of
290 osmotic activity during granule maturation (Castle & Castle, 1993). One of the
291 biological roles of salivary PRPs is to protect epithelial cells against the toxic effects of
292 food (Messana *et al.*, 2008).

293 Cystatins act as an inhibitor of cysteine proteinase and belong to an evolutionally
294 related superfamily (Shaw & Chaparro, 1999). Cystatin S accounts for 8% of the weight
295 of salivary proteins (Messana *et al.*, 2008). Von Ebner gland proteins (*Vegp2*) also act as
296 an inhibitor of cysteine proteinase (Amerongen & Veerman, 2002). Coupled with the
297 high level of Cyss gene expression in the whey-drinking group, the expression of
298 cystatin S protein also increased in the SLGs and saliva due to whey administration.
299 Salivary cystatin S plays a role in controlling proteolytic activity, either from the host or
300 from microorganisms, to protect the oral cavity.

301 Salivary amylase accounts for approximately 20% of the weight of salivary proteins
302 (Messana *et al.*, 2008). Amylase (*Amy1a,* salivary type) was induced in the SLGs of the
303 whey-drinking group when compared to those of the water-drinking group.

304 Kallikreins, which are serine proteases, are secreted as inactive zymogen granules
305 and are activated by the cleavage of an N-terminal peptide (Yousef & Diamandis, 2003).
306 Approximately 13 kallikreins have been identified in rat. The expression of kallikrein
307 genes is tissue-specific. *Klk1,* which is mainly expressed in the salivary glands, kidney
308 and pancreas, plays a constitutive and/or developmental role in glands or renal
309 physiology (Swift *et al.*, 1982; Clements *et al.*, 1990). *Klk1* and Klk1-related peptidase
310 genes (*Klks3, Klk1c10, Klk1l, GK11, Klk1b21, Klk1c10l2*) were up-regulated by 1.6- to
311 11.5-fold in the SLGs of the whey-drinking group when compared to those of the
312 water-drinking group. Tonin (*Ton*) is also a serine proteinase from the kallikrein family;
313 it cleaves angiotensin II from angiotensin I, from the synthetic tetradecapeptide
314 corresponding to the N-terminal segment of angiotensinogen and directly from
315 angiotensinogen (Boucher *et al.*, 1977; Pacheco Dda *et al.*, 2013). There is 75% homology
316 between the sequence of the γ-subunit of nerve growth factor and tonin (Lazure *et al.*,

1981). Tonin isolated from rat submandibular glands has also been reported to be very closely related to the epidermal growth factor-binding protein (Lazure *et al.*, 1981). These results suggest that kallikrein family proteins induced by whey-administration may prevent and/or restore age-dependent atrophy of SLGs.

Androgen-binding protein (*Abpa*), which is a member of the secretoglobin family (Jackson *et al.*, 2011), is expressed in the SLGs as well as the submandibular and parotid glands, and is then secreted into the saliva (Vandewege *et al.*, 2013). The secretoglobin family has an important role in modulating anti-inflammation, tissue repair, and tumor suppression and activation (Jackson *et al.*, 2011). This family of proteins has also been reported to repair dry eye (Versura *et al.*, 2010).

The AP-2 family of transcription factors consists of five members, *AP-2α, AP-2β, AP-2γ, AP-2δ* and *AP-2ε*, each encoded by a separate gene (Eckert *et al.*, 2005). Administration of whey induced expression of the *AP-2β* gene, but not the *AP-2α, AP-2γ, AP-2δ* or *AP-2ε* genes (Supplementary table 3). AP-2 is known as a tissue-specific collaborating factor and plays a critical role in the androgen-dependent transcription program (Hu & Gallo, 2010; Pihlajamaa *et al.*, 2014).

Cyss, one of the up-regulated genes in this study, has a potential AP-2 binding site (−194, TGGGGA) and androgen receptor (AR) response element which is the same as the glucocorticoid/progesterone response element in 5'-flanking region (Shaw & Chaparro, 1999). In androgen-dependent transcription programs, AR-binding is not sufficient to activate or repress the transcription and collaborating factors are necessary as AR modulators. More than 90% of up- or down-regulated genes by whey-administration in this experiment have an AR response element and AP-2 binding site in their promoter regions, suggesting that enhancement of AR-binding and promoter binding by AP-2 regulated the expression of genes associated with salivary proteins or lipid metabolism in salivary glands.

Dietary caloric restriction manipulates aging, maintaining health, and function (Roth & Ingram, 2015). Regardless of a caloric restriction, administration of whey alters

gene expression in elderly salivary glands and then prevents and/or restores age-dependent dysfunction of salivary glands, leading to maintain systemic health.

5. Conclusion

Comprehensive analysis of genetic profiles revealed that oral administration of whey induced the expression of *Abpa* and *Tcfap2b* in SLGs of elderly rats. Androgens transported through the cytoplasm by androgen binding proteins interact with AR in nuclei. AR and AP-2 beta binding to promoter of genes encoding salivary proteins and salivary gland homeostasis initiates the transcriptional responses. Through this process, bioactive components of whey restore atrophy and dysfunction of salivary glands in aged rats. Kallikrein family including tonin may prevent and/or restore age-dependent atrophy of SLGs. To our knowledge, this is the first study to report that whey-administration alters gene expression, thereby preventing and/or restoring age-dependent atrophy and functional decline in the salivary glands of aged rats.

Acknowledgements

This work was supported by a Grant-in-Aid from the Shikoku Bureau of the Ministry of Economy, Trade and Industry, and by “A-STEP” and “Program to Supporting Research Activities of Female Researchers” from Japan Science and Technology Agency. We are grateful to Professor J.D. Castle for providing anti-proline-rich protein antibody and APRO Life Science Institute Inc. (Tokushima, Japan) for holding meetings.

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Figure Legends

Fig. 1. Effect of whey- or water-drinking on the morphology of the sublingual glands (SLGs). Eight-week-old rats were given water (A) or whey (B) for 1 month. Eighty-four-week-old rats were given water (C) or whey (D) for 1 month. Bar, 10 μm.

Fig. 2. Venn diagram and scatterplot analysis. Using a Venn diagram (A), overlapping genes were analyzed among four sets. One set comprised microarray data from a whey-drinking elderly rat (OJ) vs. those from a water-drinking elderly rat (OC). Scatterplot analysis (B) of genes that were differentially expressed by more than 1.5-fold between the whey-drinking group and the water-drinking group. Four separate experiments were performed. In B-a, B-b, B-c, and B-d there were 512, 238, 473, and 938 entities, respectively, with more than a 1.5-fold difference between the whey-drinking group and the water-drinking group.

Fig.3. Effects of whey-drinking on the mRNA levels analyzed by qRT-PCR. The total RNA obtained from SLGs from 84-week-old rats given water or whey. Mean values with standard errors of relative mRNA expression compared to water-drinking group are shown. n=3, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

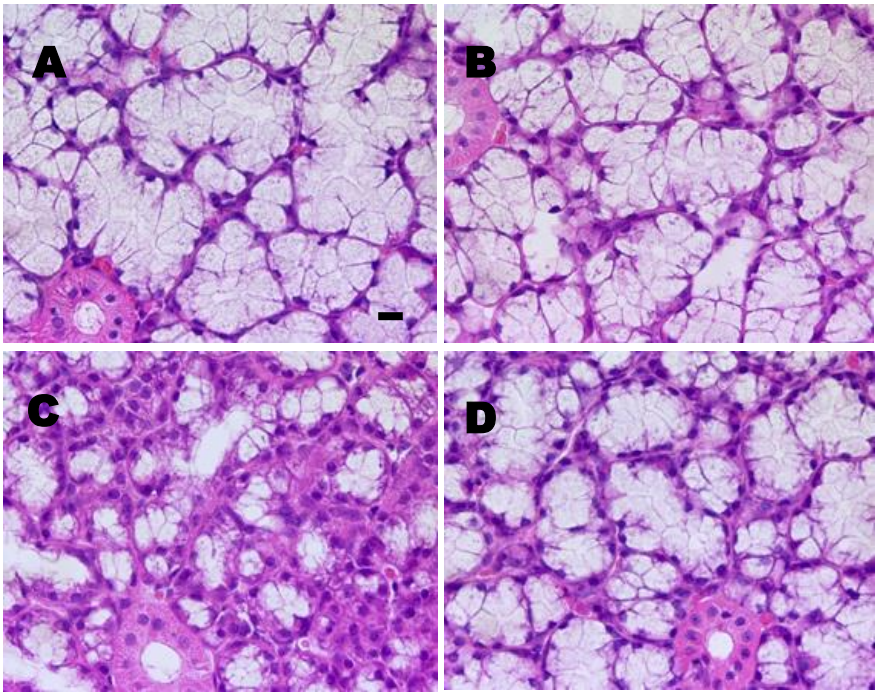
Fig.4. Effects of whey-drinking on proline-rich protein (PRP), cystatin S, and amylase levels in SLGs and saliva. SLGs (A) and saliva (B) samples were treated with Laemmli sample buffer and SLGs sample with 15 μg of protein or 15 μl saliva were subjected to SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and immunoblotted with anti-PRP antibody (a), anti-cystatin S antibody (b), and anti-amylase antibody (c). A typical western blot is shown in photography. Densitometric analysis was expressed in graphs as the relative amount of chemiluminescence in whey-drinking group vs. water-drinking. Mean values with

535 of three separate experiments standard error were shown. n=3, *p<0.05, **p<0.01,

536 ***p<0.001.

537

FIG.1



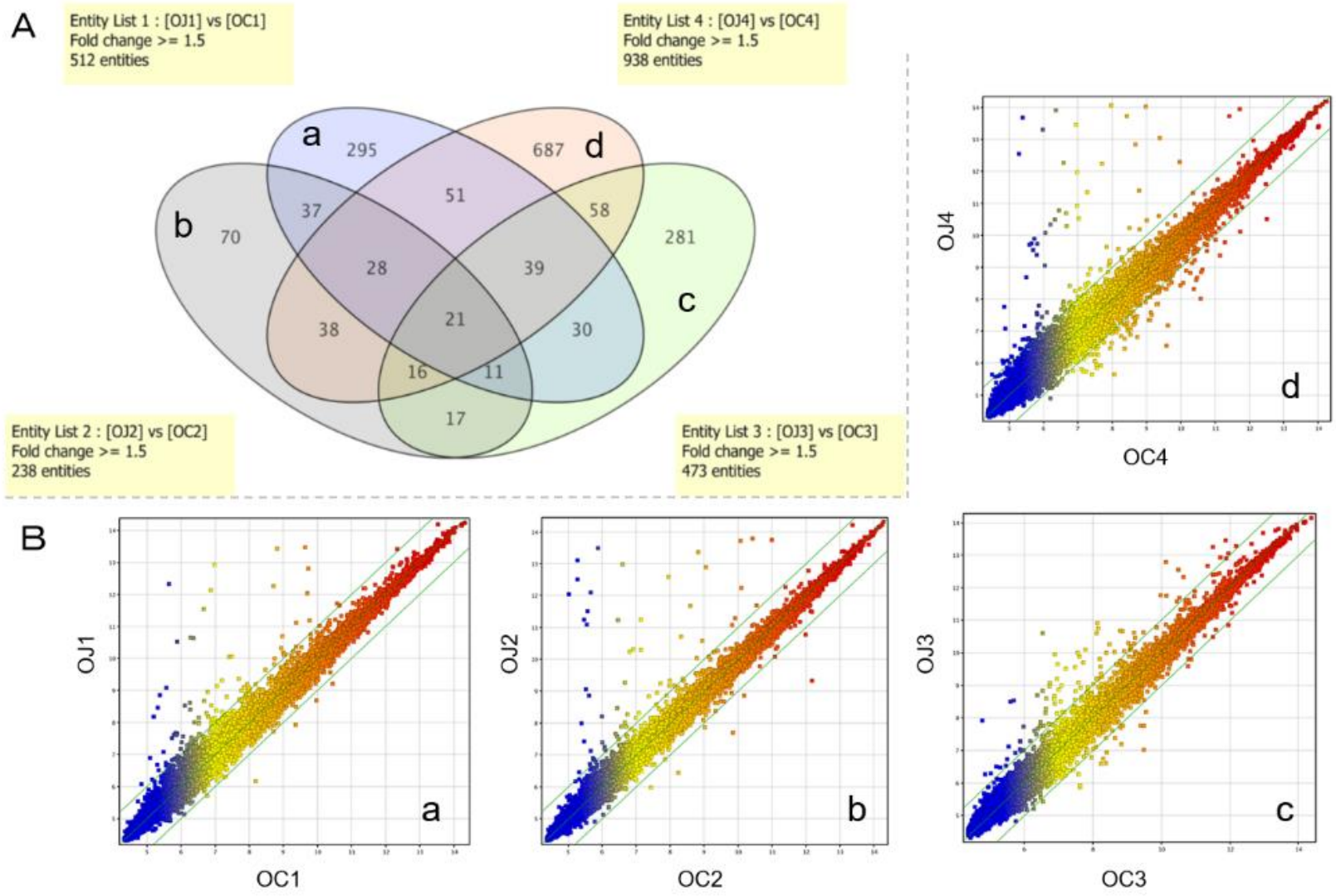

A
Entity List 1 : [OJ1] vs [OC1]
Fold change >= 1.5
512 entities
Entity List 4 : [OJ4] vs [OC4]
Fold change >= 1.5
938 entities
a
b
c
d
295
687
70
281
37
51
58
28
39
38
21
30
16
11
17
Entity List 2 : [OJ2] vs [OC2]
Fold change >= 1.5
238 entities
Entity List 3 : [OJ3] vs [OC3]
Fold change >= 1.5
473 entities
OJ4
OC4
B
OJ1
OC1
OJ2
OC2
OJ3
OC3

FIG.2

FIG. 3

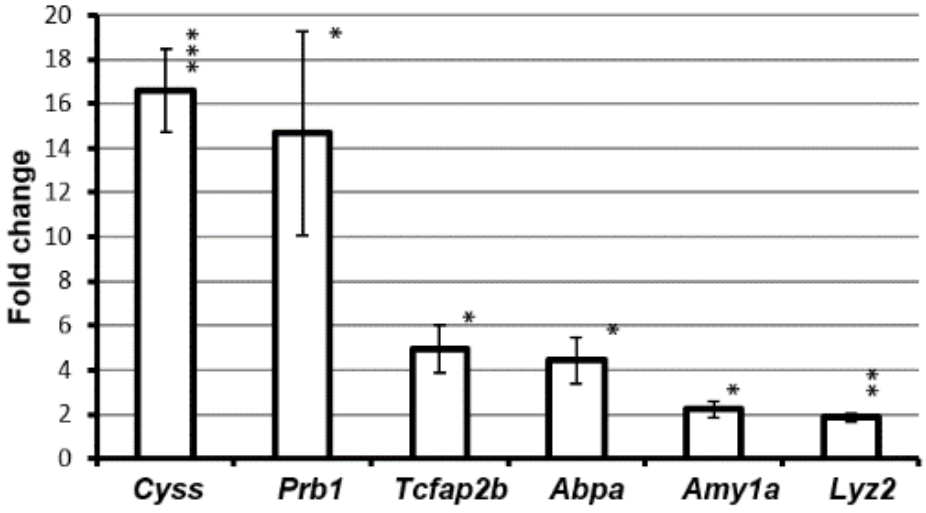

Fold change
20
18
16
14
12
10
8
6
4
2
0
Cyss
Prb1
Tcfap2b
Abpa
Amy1a
Lyz2

FIG.4

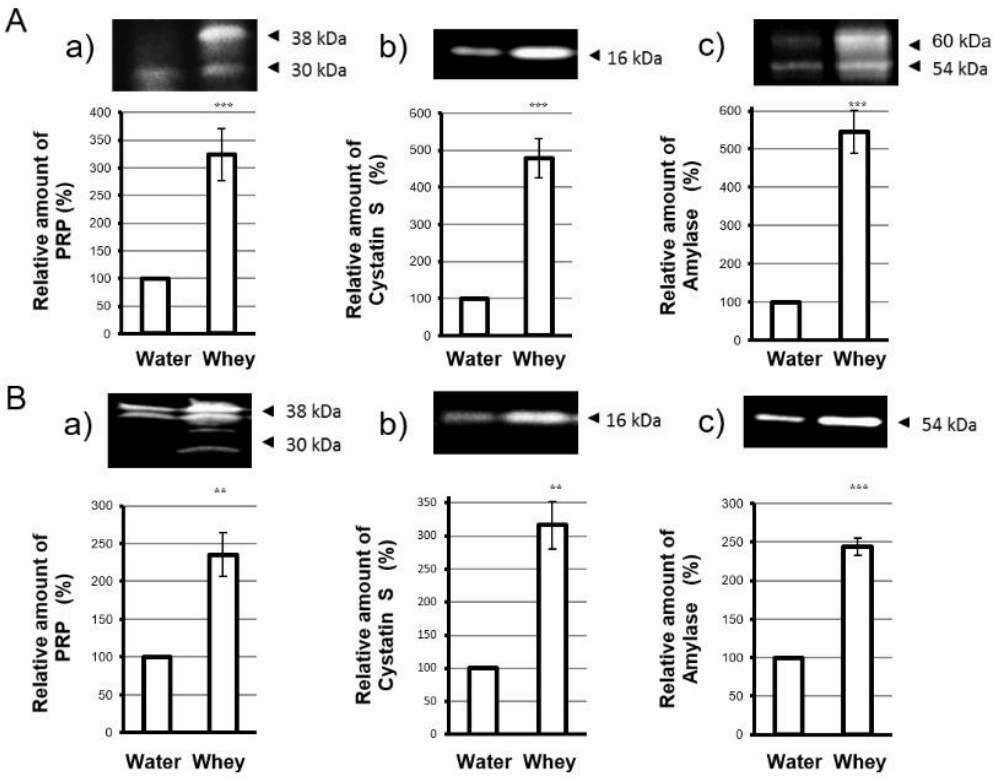

A
a)
38 kDa
30 kDa
b)
16 kDa
c)
60 kDa
54 kDa
Relative amount of PRP (%)
Relative amount of Cystatin S (%)
Relative amount of Amylase (%)
Water Whey
B
a)
38 kDa
30 kDa
b)
16 kDa
c)
54 kDa
Relative amount of PRP (%)
Relative amount of Cystatin S (%)
Relative amount of Amylase (%)
Water Whey

Table 1. Body weight, drinking volume, salivary gland weight, salivary volume and salivary protein concentration levels in water-drinking group and whey-drinking group.

		Young rats		Elderly rats	
		Water	Whey	Water	Whey
Body weight	Initial	293±3 (12-week)	294±6 (12-week)	494±5 (84-week)	489±5 (84-week)
	Final	327±17 (16-week)	315±1 (16-week)	489±5 (88-week)	479±2 (88-week)
Drinking volume (g/day)		22.3±0.5	24.1±1.4	25.7±0.8	27.1±0.5
Salivary gland weight (mg)	Parotid gland	196±4	197±8	152±7	149±7
	Submandibular gland	255±5	252±8	252±10	273±6
	Sublingual gland	48.4±4.5	48.5±0.8	46.0±1.2	54.9±1.7
Salivary volume (mg/10-20min)		296±13	345±15	180±12	313±6
Salivary protein concentration (mg/ml)		9.67±0.11	9.50±0.09	7.64±0.68	9.99±0.46

Data represent means ± SE (n=6).

*p<0.05, **p<0.01

Table 2. Genes up-regulated by >1.5-fold in SLGs from whey-drinking rats compared to those from water-drinking rats

Genedescription	Genesymbol	Fold change	Accession no.	*p*-value	FDR
Major salivary protein genes					
Proline-rich proteins					
proline rich, lacrimal 1	Prol1	8.66	NM_133513	0.035	0.102#
proline rich protein 2	Prp2	7.20	NM_001013211	0.060	0.102#
proline-rich proteoglycan 1	Prpg1	3.06	NM_172064	0.017	0.102#
proline-rich protein 15	Prp15	2.81	NM_012632	0.033	0.102#
proline-rich protein BstNI subfamily 1	Prb1	1.72	M83567	0.175	0.196
Cystatin proteinase inhibitor					
cystatin S	Cyss	25.85	NM_198685	0.003	0.054#
von Ebners gland protein 2	Vegp2	2.16	NM_053574	0.075	0.103#
Amylase					
amylase, alpha 1A (salivary)	Amy1a	3.27	NM_001010970	0.056	0.102#
Salivary gland homeostasis protein genes					
Serine endopeptidases					
tonin	Ton	15.66	NM_012677	0.052	0.102#
kallikrein, submaxillary gland S3	Klks3	11.44	NM_175759	0.060	0.102#
T-kininogenase	Klk1c10	10.63	NM_001135173	0.071	0.102#
kallikrein 1	Klk1	6.90	NM_001005382	0.062	0.102#
kallikrein 1-like peptidase	Klk1l	3.92	NM_012593	0.041	0.102#
glandular kallikrein 11	Gk11	2.81	NM_001003977	0.142	0.162
kallikrein 1-related peptidase b21	Klk1b21	2.17	NM_001013067	0.050	0.102#
kallikrein 1-related peptidase c10-like 2	Klk1c10l2	1.62	L33840	0.069	0.102#
Androgen related protein					
submaxillary gland androgen regulated protein 3A	Smr3a	10.68	NM_001017497	0.071	0.102#
androgen binding protein, alpha	Abpa	4.98	NM_001100859	0.035	0.102#
ABP beta	LOC494538	4.71	GU269241	0.033	0.102#

similar to androgen-binding protein eta	LOC689199	1.73	NM_001170457	0.181	0.196
androgen binding protein zeta	Abpz	1.68	GU269242	0.190	0.200
Transcriptional regulator					
transcription factor AP-2 beta	Tcfap2b	2.13	NM_001106896	0.003	0.054#
Signal transduction					
phosphodiesterase 3A, cGMP inhibited	Pde3a	1.67	NM_017337	0.002	0.054#
Minor salivary protein genes					
prolactin induced protein	Pip	6.38	NM_022708	0.035	0.102#
glutamine/glutamic acid-rich protein A	Grpca	5.91	NM_181440	0.055	0.102#
cytidine monophosphate-N-acetylneuraminic acid hydroxylase	Cmah	4.96	NM_001024273	0.079	0.105
alpha-2u globulin PGCL4	Obp3	4.59	NM_147215	0.050	0.102#
alpha-2u globulin PGCL2	LOC298116	4.45	NM_001033959	0.051	0.102#
alpha-2u globulin PGCL3	LOC259244	4.37	NM_147212	0.057	0.102#
salivary protein 1	Spt1	4.35	NM_019171	0.069	0.102#
major urinary protein 5	Mup5	4.35	NM_203325	0.044	0.102#
major urinary protein 4	Mup4	4.24	NM_198784	0.050	0.102#
alpha-2u globulin PGCL5	LOC259245	4.08	NM_147213	0.049	0.102#
variable coding sequence A1	Vcsa1	3.83	NM_012684	0.102	0.121
alpha2u globulin	LOC298111	3.73	NM_001024248	0.051	0.102#
variable coding sequence A2	Vcsa2	3.54	NM_198729	0.021	0.102#
alpha-2u globulin PGCL1	LOC259246	3.30	NM_147214	0.069	0.102#
ABO blood group	Abo	3.04	NM_023094	0.088	0.112
chemokine (C-X-C motif) ligand 13	Cxcl13	1.63	NM_001017496	0.293	0.293
lactoperoxidase	Lpo	1.60	NM_001105829	0.010	0.102#
Other genes					
RGD1559532	RGD1559532	3.43	NM_001024983	0.036	0.102#
carbonic anhydrase 6	Car6	2.77	NM_001134841	0.020	0.102#
claudin 22	Cldn22	2.66	NM_001110143	0.081	0.106
triadin	Trdn	2.27	NM_021666	0.100	0.122
glycosylation dependent cell adhesion molecule 1	Glycam1	2.09	NM_012794	0.265	0.270

epidermal growth factor	Egf	1.91	NM_012842	0.038	0.102#
angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	Agt	1.87	NM_134432	0.194	0.201
similar to lipase-like, ab-hydrolase domain containing 2	RGD1565682	1.83	XM_008760354	0.033	0.102#
carbonic anhydrase II	Car2	1.77	NM_019291	0.051	0.102#
similar to 9530008L14Rik protein	RGD1305679	1.72	BC088287	0.023	0.102#
similar to protein of unknown function	RGD1566243	1.69	NM_001134635	0.037	0.102#
melanoma inhibitory activity	Mia	1.64	NM_030852	0.039	0.102#
solute carrier family 6 (neurotransmitter transporter)	Slc6a14	1.63	NM_001037544	0.096	0.119
solute carrier family 37 (glycerol-3-phosphate transporter)	Slc37a2	1.55	NM_001191994	0.073	0.103#
coiled-coil domain containing 129	Ccdc129	1.53	NM_001191973	0.042	0.102#
scavenger receptor class A, member 3	Scara3	1.50	NM_001108870	0.135	0.157

#FDR≦0.1

Table 3. Genes dowm-regulated by >1.5-fold in SLGs from whey-drinking rats compared to those from water-drinking rats

Genedescription	Genesymbol	Fold change	Accession no.	p-value	FDR
Lipid metabilism related genes					
similar to fatty aldehyde dehydrogenase-like	LOC688778	-2.85	XM_008760188	0.002	0.000#
prostaglandin D receptor-like	Ptgdrl	-1.51	NM_022241	0.019	0.037#
Transcriptional regulator related genes					
forkhead box P2	Foxp2	-1.55	XM_002729284	0.003	0.013#
Cancer related genes					
hemopexin	Hpx	-1.58	NM_053318	0.072	0.090#
protease, serine, 37	Prss37	-1.52	NM_001108625	0.005	0.016#
Signal transduction genes					
synaptosomal-associated protein 25	Snap25	-2.18	NM_030991	0.118	0.131
immediate early response 3	Ier3	-1.53	NM_212505	0.007	0.016#
Immune response related genes					
mCG127631-like	LOC366766	-1.71		0.021	0.041#
pre-B lymphocyte 3-like	LOC682821	-1.51	NW_001084890	0.301	0.301
Senescence related genes					
cysteine-rich, angiogenic inducer, 61	Cyr61	-1.96	NM_031327	0.029	0.408

#FDR≦0.1

Table 4. List of overlapping genes in a Venn diagram

Genedescription	Genesymbol	Fold change	Accession no.
Secretory proteins			
cystatin S	Cyss	25.85	NM_198685
alpha-2u globulin PGCL1	LOC259246	3.30	NM_147214
alpha-2u globulin PGCL2	LOC298116	4.45	NM_001033959
alpha-2u globulin PGCL3	LOC259244	4.37	NM_147212
alpha-2u globulin PGCL4	Obp3	4.59	NM_147215
alpha-2u globulin PGCL5	LOC259245	4.08	NM_147213
major urinary protein 4	Mup4	4.24	NM_198784
major urinary protein 5	Mup5	4.35	NM_203325
Homeostasis proteins			
kallikrein 1	Klk1	6.90	NM_001005382
androgen binding protein, alpha	Abpa	4.98	NM_001100859
transcription factor AP-2 beta	Tcfap2b	2.13	NM_001106896
similar to fatty aldehyde dehydrogenase-like	LOC688778	-2.85	XM_008760188
pre-B lymphocyte 3-like	LOC682821	-1.51	NW_001084890