The effects of native whey and α-lactalbumin on the social and individual behaviour of C57BL/6J mice

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Abstract

Milk proteins are the main components of everyday feeding and demonstrate a promising potential to change the mental condition. However, the effects of milk proteins after prolonged use remain poorly understood. The aim of the present study was to compare the effects of two whey proteins (α-lactalbumin (α-lac) and native whey) with casein on social and individual behaviour in mice. During a 30 d-long dietary intervention, male C57BL/6J mice had *ad libitum* access to an experimental diet containing 17 % (w/w) of one of three protein sources: α-lac, native whey or casein. Mice had voluntary access to a running wheel. Social behaviour (group and resident–intruder activity) was tested at baseline and at the end of the intervention. Half of each dietary group was then withdrawn from the diet and running wheel for 7 d, and social activity and individual behaviour tests (open field, elevated-plus maze, light–dark box and forced swimming) were performed, to evaluate anxiety and depression-like status. The study shows that the long-term ingestion of whey proteins may modulate behaviour when compared with casein. Diet enriched with α-lac exhibited anxiolytic and antidepressive activities while the whey diet improved sociability. The differences between the diet groups were pronounced under the running wheel and the withdrawal of the experimental diet, suggesting that the beneficial effects of the milk proteins are clearer in stressful situations. Diet-induced behavioural changes remained visible for a week after feeding, which suggests that the proteins of the milk whey fraction have prolonged efficacy on the mental state of mice.

Key words: Casein: α-Lactalbumin: Native whey: Running wheels: Behaviour

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Milk-derived proteins comprise two major fractions, casein and whey proteins, that differ in their amino acid composition, absorption and bioactive properties[1,2]. The neuroactive effects of milk proteins are mostly associated with the serotonin (5-hydroxytryptamine (5-HT)) system. The neurotransmitter 5-HT is the main brain regulator of behaviour[2] and its activity is dependent mostly on the availability of tryptophan (Trp), an essential amino acid precursor of 5-HT. The enzyme tryptophan hydroxylase converts Trp to 5-HT and is responsible for the concentration and availability of Trp in the blood that increases the synthesis and release of 5-HT[2].

The whey-derived protein α-lactalbumin (α-lac) is the main source of Trp from food[3]. A meal enriched with α-lac showed beneficial anxiolytic-like effects on rats[4] and improved mood and cognition in stress-prone subjects after an acute intervention[5–8]. These changes were, however, not observed in healthy subjects, despite their increased plasma Trp concentration and ratio of Trp to other large neutral amino acids[9]. It has been shown that after a 1-week intake of a diet enriched with α-lac, basal extracellular 5-HT levels were increasingly enhanced in rats, which reflects the increased serotonergic activity[4]. Although the dietary-induced behavioural potential of whey-derived proteins appears promising, the effects of prolonged protein intake have been inadequately investigated. There are only a few studies on the effects of the chronic intake of milk protein on animal behaviour such as eating, grooming, resting, common activity[10] or energy intake[11].

Contrary to whey proteins and their α-lac component, casein, similar to many other food protein sources, has depleted Trp content when compared with large neutral amino acids. As a result, most of the previous studies have used a casein-containing diet as a control[7–9]. Thus, in the present study, we explored the beneficial effects of α-lac-containing whey proteins such as α-lac and native whey (whey) when compared with casein. In addition to α-lac, native whey consists of β-lactoglobulin and lactoferrin, and may thus have behavioural effects beyond α-lac[12].

The aim of the present study was to determine the behavioural effects of long-term diets containing whey proteins (α-lac or whey) when compared with casein-induced effects in C57BL/6J mice. Mouse housing was enriched with a running wheel to prevent any dairy diet-induced weight gain[13]. It has been shown that voluntary physical exercise has an ability to activate central dopamine, noradrenaline and serotonin systems[14], and to improve body composition in combination with amino acid food supplementation[15]. In the present study, mouse social behaviour was estimated after a 30 d protein intervention. Then, each diet group was subdivided into two subgroups: mice that continued to receive the experimental diet and access to the running wheel (enriched housing condition) and mice that returned to standard laboratory conditions (standard housing conditions) for 7 d. Social and individual behaviour was estimated to specify the delayed effects of the prolonged diet.

Materials and methods

Animals

Adult C57BL/6J male mice (Charles River) of 2·5 months old were housed individually in standard plastic cages (20 × 34 × 15 cm) equipped with a wireless running wheel (ENV-044 model; Med Associates, Inc.), a sleeping shelter and a food cup. The animals lived under a 12 h daylight cycle with lights on at 06.00 hours under temperature- and humidity-controlled conditions. Running-wheel revolutions (n) were recorded by Wi-Fi technology. Food consumption and body weight were measured every 2nd day. All procedures were approved by the National Animal Experiment Board and carried out in accordance with the guidelines for experimental animal care (ESLH-2008-01 090/Ym-23).

Experimental diets

For the present study, three experimental diets containing 17 % (w/w) protein were applied for 30 d after a 3 d habituation period during which mice were fed on a standard laboratory rodent chow (Harlan rodent diet 2018; Harlan BV). The nutritional content of the experimental diets is presented in Table 1. The α-lac group received a diet containing α-lac (92·7 % (w/w) of the final product; Valio Limited) and the whey group received a diet containing native whey (89·1 % (w/w) of the final product in which 23·9 % was α-lac and 65·2 % was β-lactoglobulin). Both whey proteins were mixed with a protein-free premix (commercial D09041501M powder; Research Diets, Inc.). As a control, we used a casein protein diet (commercial D12450BM powder; Research Diets, Inc.).

The protein powder was mixed with water to minimise powder spillage and to provide a uniform dough-like texture[16]. A fresh chow portion was prepared every 4th day and kept at −20°C before use. The fresh portion corresponding to the amount to cover normal *ad libitum* consumption (6–7 g) in mice[17] was served every 2nd day. Water was available *ad libitum*.

Table 1. Nutritional content of the experimental diets (g/100 g)

	Casein	α-Lac	Whey
Energy (kJ)	1577	1586	1586
Carbohydrate (g)	67·6	67·6	67·6
Fibre (g)	4·7	4·7	4·7
Proteins (g)			
Casein	19·0	0·0	0·0
L-Cystine	0·3	0·0	0·0
α-Lac	0·0	18·8	0·0
Native whey	0·0	0·0	18·8
Fat (g)	4·3	4·3	4·3
Minerals (mg)			
Ca	0·6	0·6	0·7
P	0·4	0·4	0·5
K	0·6	0·6	0·6
Mg	0·1	0·1	0·1
Na	0·1	0·3	0·1

α-Lac, α-lactalbumin.

A food preference test was done on the 24th day of dietary feeding after a 6 h food deprivation that was used to stimulate mouse exploratory activity[18]. The test was done in the environment of a T-maze apparatus[19] that was made up of grey plastic and consisted of three arms (50 × 10 cm) perpendicular to each other and with 15 cm walls. Each arm had a remote-controlled door that separated 12 cm compartments (in one arm) or 24 cm compartments (in two other arms). The animal was put in the central part (starting zone) of the apparatus for a 3 min habituation period when all doors were in the closed position so that the animal could only smell casein, α-lac or whey food pieces (2 g pellet) through the narrow door slit (5 mm). Then, the doors were opened for the next 4 min and the animal could visit all compartments and taste the pellets.

The same sizes of nearest space about the pellets were specified on the arena template as pellet-related zones and were equidistant from the starting zone. The spatial disposition of the zones was changed randomly for every animal to exclude the spatial preference. Mouse track in the T-maze was observed by a video-tracking system with EthoVision Color-Pro 3.1 software (Noldus Information Technology) to estimate which zone was the most visited. Additionally, the food preference test included the group-housed C57BL/6J males of the same age (n 10) but using only a standard laboratory food (Harlan rodent diet 2018; Harlan BV).

Study design

The study design is illustrated in Fig. 1. Mice were divided into three equal groups according to the K-means analysis (SPSS version 20.0; SPSS Institute). Each group contained a similar number of animals having different ranks to equalise individual behavioural deviations of individual locomotor activity and body weight. Spontaneous horizontal locomotor activity was tested automatically for 30 min on the arena of individual plastic rectangular cages (34 × 19 × 15 cm) using a video-tracking system with EthoVision Color-Pro 3.1 software (Noldus Information Technology). After the ranking, mice were housed individually for 3 d habituation. Then, baseline social activity was estimated as described below. A wireless running wheel
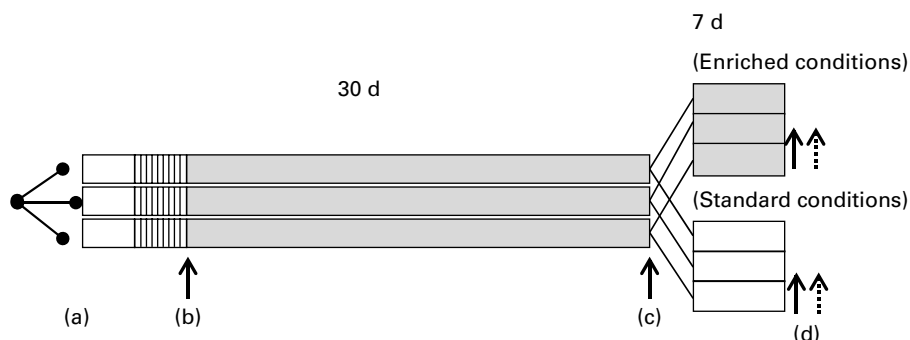
Fig. 1. Experimental design. (a) C57BL/6J mice were divided into three groups according to their weight and locomotor activity. The same protocol was used in all the diet groups (α-lactalbumin (α-lac), whey and casein). After 3 d of habituation in isolation, the running wheel was added to the cage. Social activity was tested at (b) baseline and (c) after the 30 d dietary feeding. (d) After further dividing the diet groups into two subgroups: social and individual behaviour as well as 6 h running-wheel activity were tested after 7 d. □, Standard diet, no running wheel (standard conditions); ▥, standard diet, running wheel; ▪, experimental diet (α-lac, whey or casein), running wheel (enriched conditions); ↑, social behaviour tests (group and resident–intruder tests); ↑ (dashed), individual behaviour tests (light–dark box, open field, elevated-plus maze and forced swimming).

(ENV-044 model; Med Associates, Inc.) was added into the mouse home cage in 3 d before the dietary intervention was started for the next 30 d.

After the 30 d dietary intervention, the social behaviour tests were repeated and each diet group (α-lac, whey and casein) was subdivided into two subgroups: mice that continued to use the diet and running wheel (enriched housing condition) and mice that returned to standard laboratory conditions (standard housing conditions) for 7 d. We suggested that the 7 d post-intervention phase will be useful to estimate the delayed effects of the prolonged diet intervention. Standard laboratory conditions implied a return to the standard rodent chow (Harlan rodent diet 2018; Harlan BV) and no access to the running wheel. Thus, six groups were formed at this phase of the experiment.

After 7 d, the social and individual behaviour tests were performed and the comparisons were made between the diet groups in the same conditions, but also within a diet group between the standard and enriched housing conditions. On the next day after the last behavioural test, mice in the standard housing conditions received access to the running wheel for 6 h. Their running-wheel activity was compared with the activity that was developed by mice in the enriched housing conditions. The test was done to estimate whether removal of the running wheel was significant to increase its use after returning. Behavioural measurements were done automatically or video recorded. To provide a blind test of the observation, all behavioural samples (tracks or videos) were encoded to analyse them and opened only to perform a statistical analysis.

Social behavioural tests

Mouse social activity was evaluated among other males on a new territory (group social activity) as well as towards an unknown partner (male and female) on their own territory (resident–intruder test)[20] for 9 min. Video-recorded samples were analysed subsequently by the data acquisition program Ethograph 2.06 (Ritec) according to the developed protocol[21]. Social activity tests, except the resident–intruder test towards an unknown female, were repeated three times: at

baseline; after the dietary intervention; after the 7 d post-intervention phase. The resident–intruder test towards an unknown female was done twice only: at baseline and after the dietary intervention.

The group test was performed to estimate mouse behaviour on the neutral territory of standard plastic cages (20 × 34 × 15 cm) towards other members of the temporarily organised group (three or four members). Observation was specified for five behavioural categories: (1) individual behaviour (any individual on-site behaviour, when contacts with other members are absent); (2) locomotor (any movements from site to site); (3) social contacts (any non-aggressive physical contact with the other group members); (4) consummate aggression (throws, fighting, biting and boxing); (5) defence (vertical upright, sideway stances, back defensive posture and freezing)[22].

The resident–intruder test towards an unknown male was done on the territory of housing resident cage, where an unknown male was introduced. Resident behaviour was specified for five categories: (1) individual behaviour (any behaviour when contacts with the intruder were absent); (2) social contacts (any non-aggressive physical contacts with the intruder); (3) ambivalent aggression (demonstration of aggressive intentions: spinning about the intruder, tail rattling and digging of sawdust); (4) consummate aggression (throws, fighting, biting and boxing); (5) defence (vertical upright, sideway stances, back defensive posture and freezing)[22].

The resident–intruder test towards an unknown receptive female was done in the resident territory where a female was introduced under a turned-over perforated metallic cylinder ($h = 12$ cm, base $d = 10$ cm; Etola Group) to protect the female from direct contact with the resident. The resident was allowed to sniff but not to mark or touch the female[23]. For this purpose, group-housing C57BL/6J female peers on the oestrous phase of the oestrus cycle were used (n 10). The phase of the oestrous cycle was determined as described previously[24] on the test day. The intruder behaviour was specified for eight categories: (1) individual behaviour (any behaviour demonstrating a great distance away (more than 3 cm) from the cylinder); (2) sitting near the cylinder (less

than 3 cm); (3) running about the cylinder; (4) sniffing through the holes of the cylinder; (5) climbing on the cylinder; (6) sitting on top of the cylinder; (7) digging under the cylinder; (8) shifting or touching the cylinder by forepaws.

Individual behavioural tests

The test battery includes light–dark box, elevated-plus-maze and open-field tests[25,26] that are based on the natural conflict between the tendencies to explore a new environment and to avoid potentially dangerous (open and lighted) areas[27]. Depression-like status was evaluated with a forced swimming test[28]. The individual behaviour tests were performed after the 7 d post-intervention phase.

The light–dark box test was performed in a transparent plastic cage ($33 \times 55 \times 19$ cm) divided into open-lighted (33×33 cm) and dark (33×22 cm) parts that connect through a shelf. The mouse was placed in the centre of the open-lighted area for 5 min and the duration and distance of the movement on the lighted area as well as the number of entries into the dark area were recorded automatically by a video-tracking system (EthoVision; Noldus Information Technology).

The elevated-plus-maze test was performed the day after the light–dark box test. The maze apparatus was elevated to 50 cm above the floor level and consisted of the central start platform (5×5 cm), from which two open arms (5×40 cm with a 0·7 cm margin) and two closed arms (5×40 cm with 20 cm walls) extended in opposite directions. Mouse track was recorded for 5 min by a video-tracking system (EthoVision) to measure the time spent in the open arms and in the protected zone (closed arms and start platform).

The open-field test was performed for 6 min immediately after the elevated-plus-maze test in a transparent plastic cage ($33 \times 55 \times 19$ cm) in which the floor was divided into fifteen squares (11×11 cm) to specify the central and peripheral zones. The test was subdivided into two parts. Initially, the animal was put in the centre of the field for 3 min and then a yellow plastic ball (4 cm in diameter) was quickly installed in the central square. Mouse behaviour after the installation of the new object was observed for the next 3 min. Videos were analysed subsequently using computer-assisted data acquisition software (Ethograph 2.06) according to the developed protocol[22,25]. Behaviour was specified as central and peripheral locomotor and non-locomotor behaviour; the new object exploration includes: sniffing; touching; manipulation by forepaws; freezing.

The forced swimming test was performed as the last test to evaluate mouse depression-like status that is based on the measure of immobility[28]. Mice were individually placed in a glass cylinder (26·5 cm in height, 17 cm in diameter) filled with water ($22 \pm 1°$C) to 16 cm for 6 min. Mouse swimming behaviour (immobility events when the mouse froze; climbing, swimming and floating) was videotaped and measured by Ethograph 2.06 and EthoVision[29].

Statistical analysis

Statistical analyses were conducted using the predictive analytics software PASW Statistics 20.0 (SPSS, Inc.). Primary comparisons of the results were done to compare the effects of the whey proteins with casein-induced effects under housing conditions. Multivariate ANOVA (two-way) followed by a Bonferroni or Kruskal–Wallis non-parametrical post hoc test ($P<0.05$) was used to analyse the data obtained from the social, food preference, light–dark box, elevated-plus-maze and forced swimming tests. If the tests were repeated several times (social tests), analysis for repeated measurements followed by a Bonferroni post hoc test ($P<0.05$) was applied. P values of less than 0·05 were considered to be statistically significant. Kaplan–Meier survival analysis with a Mantel–Cox non-parametric test ($P<0.05$) was used to analyse the probability of the first outcome of the element (latency of aggression and latency of immobility) that was based on the analysis of the time intervals[30].

Behavioural elements specified for the analysis or combined group of the elements (category) received by Ethograph 2.06 were presented for statistical analysis in five measurements: total duration (sum of the duration of the element for the test); relative duration (ratio of the total element duration to the test duration); medial duration (ratio of the element duration to its total frequency); total frequency; relative frequency (ratio of the element frequency to the sum of all frequencies of the observed elements).

Numerous behavioural observations, as a matrix of probabilistic changes, require special statistical analysis of a higher level than mere descriptive statistics to evaluate all data as an integrated framework[31]. A discriminant function analysis, a multivariate statistical technique (based on Mahalanobis distance and a stepwise forward procedure), was used with the purpose to: (1) distinguish the dietary groups according to their behavioural function; (2) identify discriminate variables that contribute significantly to group differences; (3) determine an optimal manner for distinguishing between the groups; (4) determine unclassified individuals[32]. The analysis suggests that the hypothetical linear n-component functions ($F = dfc_1 x\ v_1 + \ldots + dfc_n x\ v_n$) could be composed to describe a mouse behavioural profile. In this function coefficients ($dfc_1 x$) were calculated by discriminant function analysis based on individual and social behavioural measurements and v refers to behavioural elements.

Results

Food preference

All the tested mice spent more time in the α-lac zone than in the casein zone ($F_{6,236} = 2.61$, $P=0.02$). Especially, the α-lac- and whey-fed mice preferred the α-lac zone when compared with the casein-fed ones (Kruskal–Wallis non-parametric test, $P=0.045$). The whey zone was also preferred more than the casein zone, except for the whey-fed mice.

Food consumption and weight gain

All the dietary groups demonstrated identical food consumption ($F_{2,110} = 3.05$, NS; Fig. 2(a)) while their body weight increased significantly ($F_{2,110} = 8.26$, $P<0.0001$; Fig. 2(b)):
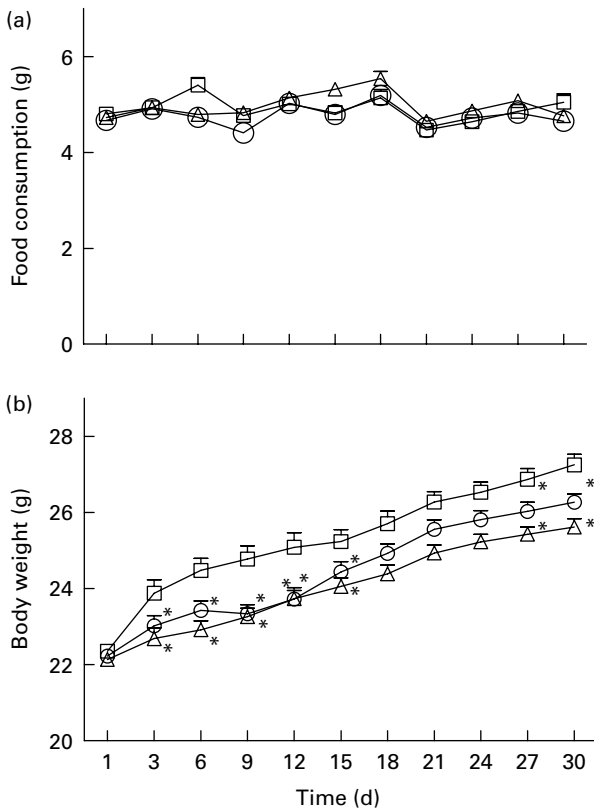
Fig. 2. (a) Food consumption and (b) body weight measured during the 30 d dietary feeding of casein (n 32, –□–), whey (n 42, –○–) and α-lactalbumin (n 41, –△–) on every 3rd day. Values are means, with their standard errors represented by vertical bars. * Mean value was significantly different when compared with casein (P<0·05, Bonferroni post hoc test for repeated measurements).

whey- and α-lac-fed mice increased their body weight less than the casein-fed ones.

Running-wheel activity

Running-wheel activity between the dietary groups from day to day during the 30 d dietary intervention was similar ($F_{2,233} = 1·01$, NS) as well as its circadian distribution. The 6 h running-wheel test after the 7 d post-intervention revealed that the casein group under the standard housing conditions increased running-wheel activity when compared with casein-fed mice in the enriched conditions ($F_{1,38} = 6·81$, $P=0·014$).

Social behavioural tests

At baseline, there were no differences in group social activity between the dietary groups. After the 30 d diet, whey-fed mice demonstrated more often non-aggressive contacts (relative frequency: $F_{2,39} = 4·74$, $P=0·015$), individual behaviour (relative frequency: $F_{2,39} = 4·95$, $P=0·012$) and locomotor behaviour (relative frequency: $F_{2,39} = 4·70$, $P=0·015$) than the casein and α-lac groups. Whey-fed mice changed their behaviour more often, suggesting that they were more dynamic. There were no differences in aggression behaviour, which was observed in 29 % of α-lac- and whey-fed mice and in 58 % of casein-fed mice.

After the 7 d post-intervention phase, no differences were found in the enriched conditions between the diet groups (Table 2). In the standard conditions, the group on the α-lac

Table 2. Social group behaviour of mice in the temporarily organised groups after the 7 d post-intervention in the different conditions

(Mean values with their standard errors)

| Behaviour | Enriched conditions | | | | | | Standard conditions | | | | | |
| | Casein | | α-Lac | | Whey | | Post-casein | | Post-α-lac | | Post-whey | |
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Individual												
Relative duration	25·9	2·9	25·4	2·7	28·3	2·7	23·6	2·9	32·5	2·7	31·2	2·7
Medial duration	2·1	0·3	1·8	0·2	2·1	0·2	2	0·3	3·1*†‡	0·2	2	0·2
Relative frequency	0·6	0·1	0·7	0·1	0·7	0·1	0·6	0·1	0·6	0·1	0·8	0·1
Locomotor												
Relative duration	11·3	1·6	10·1	1·1	12·3	1·1	11·2	1·6	10·3	1·1	12	1·1
Medial duration	0·7	0·5	0·6	0·5	0·7	0·1	0·7	0·5	0·8*	0·5	0·6	0·1
Relative frequency	0·8	0·1	0·8	0·1	0·9	0·1	0·8	0·1	0·7	0·1	0·9	0·1
Non-aggressive contacts												
Relative duration	31·7	3·8	38·9	3·5	33·5	3·5	36	3·8	20·6*†	3·5	27·9	3·5
Medial duration	4	0·5	4·1	0·4	3·5	0·4	4·1	0·5	2·7	0·4	2·8	0·4
Relative frequency	0·4	0	0·5	0	0·5	0	0·4	0	0·4*‡	0	0·5	0
Consummate aggression												
Relative duration	5·6	1·7	0·5	1·6	0·8	1·6	4·1	1·7	4·8	1·6	3·7	1·6
Medial duration	6·7	1·4	1·5	1·3	1·4	1·3	2·1	1·4	2·1	1·3	3·1	1·3
Relative frequency	0	0	0	0	0	0	0·1	0	0·1	0	0	0
Defence												
Relative duration	0·2	1·6	0	1·5	0·1	1·5	0·1	1·6	6·7	1·5	0	1·5
Medial duration	1·5	0·6	0·1	0·6	0·3	0·6	0·2	0·6	2·2*‡	0·6	0·2	0·6
Relative frequency	0	0	0	0	0	0	0	0	0·1*†	0	0	0

α-Lac, α-lactalbumin.
* Mean values were significantly different between the enriched and standard conditions for the same diet group (P<0·05).
† Mean values were significantly different compared with the casein group (P<0·05).
‡ Mean values were significantly different compared with the whey group within the same conditions (P<0·05).

diet showed low non-aggressive social activity (relative duration: $F_{2,39} = 4\cdot79$, $P=0\cdot015$), but longer periods of individual (medial duration: $F_{2,39} = 5\cdot84$, $P=0\cdot015$), locomotor (medial duration: $F_{2,39} = 4\cdot73$, $P=0\cdot015$) and defensive (medial duration: $F_{2,39} = 4\cdot42$, $P=0\cdot020$) behaviour than the casein group. Whey-fed mice in the standard conditions started a fight later than casein-fed mice (Kaplan–Meier survival analysis: log-rank Mantel–Cox $5\cdot42$, $P=0\cdot020$; Fig. 3).

When comparing the housing conditions, α-lac-fed mice only contacted less with the other group members in the standard conditions (relative frequency: $F_{2,39} = 3\cdot48$, $P=0\cdot42$), but more often demonstrated agonistic behaviour (defensive behaviour: $F_{2,39} = 4\cdot84$, $P=0\cdot014$ and aggressive behaviour: $F_{2,39} = 7\cdot05$, $P=0\cdot012$) than their peers in the enriched

conditions. Kaplan–Meier analysis showed that α-lac-fed mice in the standard conditions started a fight more quickly than their peers in the enriched conditions (log-rank Mantel–Cox $8\cdot06$, $P=0\cdot005$).

The resident–intruder test towards an unknown male was repeated three times. The baseline activity of the resident after the short-term 3 d social isolation did not contain any aggressive interactions. After 30 d isolation by the dietary intervention, aggressive behaviour was similar in all the diet groups: only 58 % of casein-fed mice and 57 % of α-lac- and whey-fed mice showed aggressive behaviour. Also, no diet-specific differences were found after the 7 d post-intervention period. However, aggressive demonstrations were observed in more animals (χ^2 test, $P=0\cdot016$) under the enriched conditions (64, 63 and 73 % of casein-, α-lac- and whey-fed mice, respectively) than after the standard conditions (16, 45 and 36 % of casein-, α-lac- and whey-fed mice, respectively). Despite the varying level of aggression under the housing conditions, any diet-specific effects remained unclear.

The resident–intruder test towards an unknown female was repeated two times. At baseline, all males demonstrated a similar concerned behaviour towards the female hidden under the cylinder. In the second test, after the 30 d dietary intervention, whey-fed mice demonstrated more activity towards the female than the casein-fed ones. Thus, whey-fed mice dug under the cylinder longer (total duration: $F_{2,39} = 3\cdot48$, $P=0\cdot041$ and relative frequency: $F_{2,39} = 4\cdot25$, $P=0\cdot022$).

Individual behavioural tests

The results of the individual behaviour tests, performed after the 7 d post-intervention phase, are presented in Table 3. In the enriched conditions, the light–dark box test showed that α-lac-fed mice spent less time and were less mobile in the light compartment than casein-fed mice ($F_{2,73} = 5\cdot65$, $P=0\cdot005$ and $F_{2,73} = 3\cdot88$, $P=0\cdot03$, respectively). However, in the standard conditions, all mice spent more time in the light compartment than their peers under the enriched conditions ($F_{1,73} = 36\cdot34$, $P<0\cdot0001$), and α-lac-fed mice also moved more actively in the light compartment ($F_{1,73} = 12\cdot89$, $P=0\cdot001$).

The elevated-plus-maze test revealed that, in the enriched conditions, the α-lac and whey groups overcame longer distance in the protected zones (closed arms and start square) when compared with the casein group in the same conditions and when compared with their peers in the standard conditions ($F_{2,73} = 4\cdot14$, $P=0\cdot027$; $F_{1,73} = 22\cdot46$, $P<0\cdot0001$ for both diet and housing conditions).

In the open-field test, diet- and housing-related effects were found after the new object was installed (for the second 3 min interval). α-Lac-fed mice in the standard conditions moved more actively on the periphery of the field ($F_{1,71} = 11\cdot48$, $P<0\cdot0001$ and $F_{1,71} = 9\cdot89$, $P=0\cdot002$ for total duration_2 and total frequency_2, respectively) and on the centre ($F_{1,71} = 5\cdot83$, $P=0\cdot0019$ and $F_{1,71} = 10\cdot24$, $P=0\cdot002$) than their peers in the enriched conditions. At the same time, whey-fed mice in the standard conditions were more mobile on the centre ($F_{1,71} = 9\cdot89$, $P=0\cdot0002$ and $F_{1,71} = 10\cdot24$, $P=0\cdot002$).
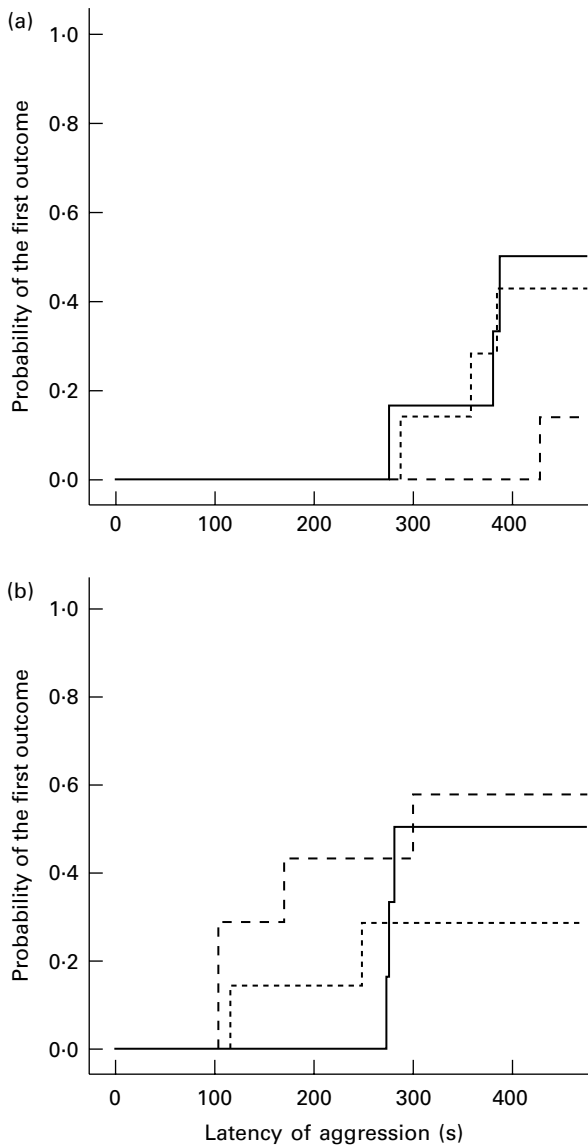

Fig. 3. Probability of the first outcome of aggression in the group tests by the Kaplan–Meier survival analysis. The results are presented for the different environmental conditions: (a) enriched conditions (casein, n 14; whey, n 19; α-lactalbumin (α-lac), n 19) and (b) standard conditions (casein, n 17; whey, n 23; α-lac, n 22). (+) Latency was censored by the test time (540 s). ——, Casein; ······, whey; - - -, α-lac.

Table 3. Individual mouse running-wheel activity and behaviour in tests assessing anxiety and depression-like status in the 7 d post-intervention phase*

(Mean values with their standard errors)

	Enriched conditions						Standard conditions					
	Casein		α-Lac		Whey		Post-casein		Post-α-lac		Post-whey	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Running wheel												
6 h activity after deprivation (n)	2947	1978	7333	1832	9556	1832	9404†	2167	11 618	1832	11 061	1832
Light−dark box												
Time spent in light (s)	187	21	124‡	17	178	18	275†	18	224†	17	251†	15
Mobility in light (s)	154	15	102‡	13	133	13	184	13	162†	11	155	11
Elevated-plus maze												
Distance in protected area (mm)	997	92	1235‡	75	1235‡	75	758	78	961†	67	864†	65
Open field												
Peripheral locomotor												
Total duration	17·2	3·7	18·6	3·0	18·2	3·0	25·7	3·2	30·5†	2·9	23·5	2·6
Total frequency	18·6	3·4	19·9	2·8	19·3	2·8	21·6	2·9	30·5†	2·6	22·3	2·4
Central locomotion												
Total duration	1·5	1·0	2·2	0·8	1·3	0·8	2·5	0·8	5·1†	0·8	3·7†	0·7
Total frequency	1·6	1·0	2·4	0·8	1·7	0·8	3·1	0·9	5·2†	0·8	4·1†	0·7
Contact with the new object												
Total duration	3·4	1·5	3·1	1·2	3·8	1·2	3·8	1·3	8·8†‡	1·2	7·6†	1·7
Total frequency	3·5	1·3	3·3	1·1	3·6	1·1	5·2	1·1	7·4†	1·0	6·3	0·9
Freezing by the new object												
Total duration	8·7	2·6	15·4§	2·1	4·9	2·1	13·6	2·2	6·7†	2·0	9·8	1·8
Total frequency	5·8	1·0	8·8§	0·8	3·6	0·8	9·5†	0·9	3·8	0·8	4·8	0·7
Forced swimming												
Immobility latency (s)	61·1	10·7	61·2§	4·9	40·5	6·0	116·4†	11·6	101·0†§	13·9	79·0†‡	8·0

α-Lac, α-lactalbumin.
* Behavioural measurements are presented for the casein, α-lac and whey groups that were exposed to the experimental diet and had access to the running wheel or were exposed to the standard diet and had no access to the running wheel.
† Mean values were significantly different between the enriched and standard conditions for the same diet group ($P<0.05$).
‡ Mean values were significantly different compared with the casein group ($P<0.05$).
§ Mean values were significantly different compared with the whey group within the same conditions ($P<0.05$).

The time to explore the new object was longer both in α-lac- and whey-fed mice in the standard conditions when compared with the enriched conditions (total duration: $F_{1,71} = 10.35$, $P=0.002$). In the standard conditions, α-lac-fed mice also contacted with the new object longer (total duration: $F_{1,71} = 10.22$, $P=0.002$) and demonstrated less and shorter time of 'freezing' (total duration: $F_{2,71} = 6.86$, $P=0.002$ and total frequency: $F_{2,71} = 13.46$, $P<0.0001$, respectively) than their peers in the enriched conditions. On the contrary, the casein group demonstrated 'freezing' more often after the standard conditions than after the enriched conditions (total frequency_2: $F_{2,71} = 13.59$, $P<0.0001$).

In the forced swimming test, whey-fed mice stopped swimming more quickly than α-lac-fed mice (log-rank Mantel−Cox 3.96, $P=0.047$ due to the Kaplan−Meier survival analysis) under the enriched conditions. They also ceased swimming more quickly than casein- and α-lac-fed mice (log-rank Mantel−Cox 5.77, $P=0.016$ and log-rank Mantel−Cox 4.62, $P=0.032$, respectively, due to the Kaplan−Meier survival analysis) under the standard conditions. Swimming was observed to be longer in all mice after the standard conditions than in the enriched conditions ($F_{2,73} = 4.32$, $P=0.017$).

Discriminant function analysis

A discriminant function analysis was done in order to estimate all data as an integrated framework. Mice under the enriched conditions were classified correctly ($P<0.05$) according to the diet received in 93.8% of the original grouped cases and in 84.4% of the cross-validated grouped cases (Fig. 4(a)). Standardised canonical discriminant function coefficients are illustrated in Table 4 for behavioural variables that contributed significantly to group differences. A small number of casein and α-lac animals (12 and 8.3%, respectively) were classified as were whey-fed mice. In turn, only 75% of whey-fed mice were classified correctly: 8.3 and 16.7% of the animals were classified similar to casein- and α-lac-fed mice, respectively.

After the standard conditions for 7 d, mice were classified correctly ($P<0.05$) by diets in 97.6% of the original grouped cases and in 90.5% of the cross-validated grouped cases (Fig. 4(b)). All (100%) of the casein animals were classified correctly, whereas 6.3% of whey males were classified as α-lac-fed mice, and 20% of α-lac males were classified as casein- (13.3%) and whey-fed (6.7%) mice. Discrimination of mice between the conditions within the same diet group was done successfully ($P<0.05$) for 100, 96 and 100% of the casein, α-lac and whey groups, respectively, which suggests that the housing conditions to modulate mouse behaviour were important.

Discussion

The effects of 30 d milk protein-containing diets were studied in C57BL/6J mice to determine whether the long-term intake
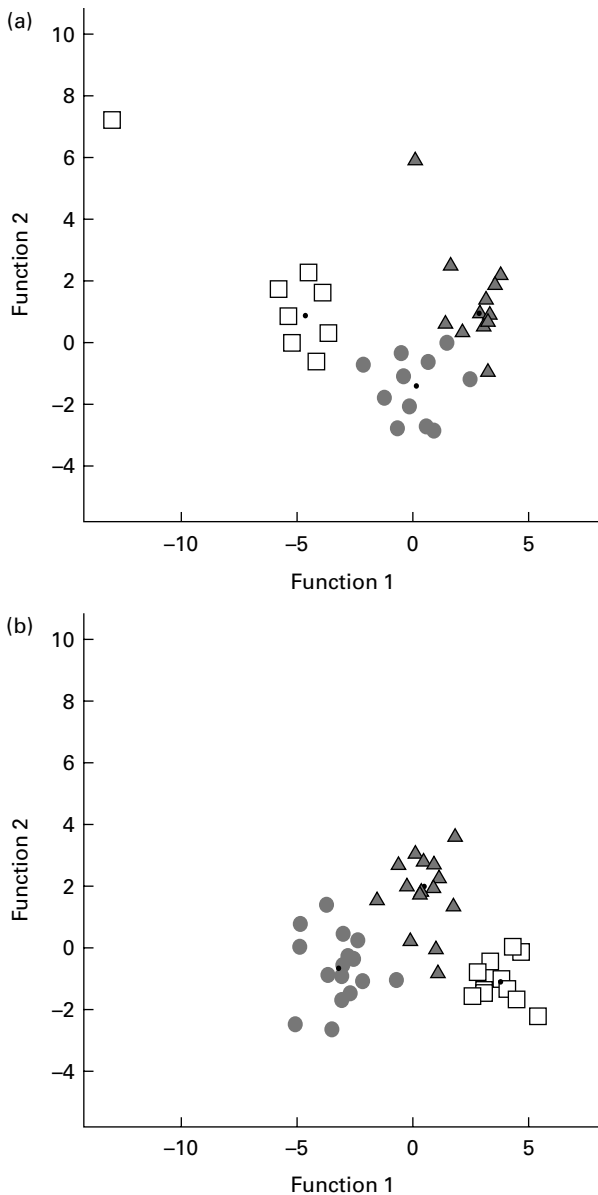
Fig. 4. Plot of the behavioural profiles based on the discriminant function analysis of the groups after the 7 d environmental changes: (a) enriched conditions or (b) standard conditions. □, Casein; ▲, α-lactalbumin; ●, whey.

of milk protein (whey proteins v. casein) affects social and individual behaviour[33]. The behavioural efficacy of milk proteins was also estimated 7 d after the diet intervention when the animals were returned to standard laboratory conditions. Physical status as a main determinant of behavioural manifestations[10] was taken care by the regular food consumption and body-weight measurements.

We found that both proteins of the milk-whey fraction, α-lac and whey, prevented weight gain. Although the exact mechanism by which these proteins affect body weight is unclear, it may be related to the α-lac component that demonstrated the ability to decrease weight in mice after diet-induced obesity[34,35]. Another pathway to body-weight regulation may be related to β-lactoglobulin, a principal

component of whey, which was found to reduce both body-weight gain and adiposity in rats after a 25 d high-protein diet[1]. In the present study, the weight-protective effects of the α-lac and whey diets were observed, despite the volume of food consumption and running-wheel activity being diet-independent and similar in all animals.

Daily running-wheel activity, as well as its circadian rhythm, remained stable throughout the experiment. This result corroborates the findings shown previously in C57BL/6J mice which held stable running-wheel activity, despite housing conditions, and may thus be regarded as a trait of the mouse line[36]. Running activity has a high rewarding potential and might compete with other positive reinforces such as ethanol[37] and drugs of abuse[36,38]. We hypothesised that if the running wheel has rewarding properties, then the returned access to the wheel after the short-term deprivation should increase the running activity. Although the activity did not differ between the diet groups after the returned access, casein-fed mice after the standard housing conditions only increased their activity when compared with their peers that continued in the enriched conditions. It might be suggested that the α-lac and whey but not casein feeding exhibited compensative, or competitive, activity.

The differences might also be found due to the decreased activity of casein-fed mice under the enriched conditions. However, we have kept in mind that the 6 h returned running-wheel test was done on the next day after the individual and social test battery, which might have also influenced the running activity. In this case, neither the α-lac nor the whey group demonstrated the changes in running activity. It means that casein anyway cannot compensate for stressful environmental effects while α-lac and whey did. At the same time, whey and α-lac had slight attractive properties according to the food preference test. It seems that food also might be considered as a competitive reward that reduced the rewarding potential of the running wheel. Orosco et al.[4] showed that an α-lac acute intervention decreased sucrose consumption, which has a high rewarding potential for mice. It seems that α-lac compensated for it due to yet unclear mechanisms.

The decreased aggression towards other males, which was expected to come up after isolation[39], might be explained by the presence of two rewarding stimuli, running wheel[40] and attractive food that, probably, strongly suppressed aggressions[41]. Whey-fed mice demonstrated more sociability in the male group and intended to contact with the hidden female more actively than casein-fed mice after the 30 d dietary intervention. Increased sexual behaviour is related usually to the increased blood testosterone level that was observed in CBA mice in the same behavioural situation[42]. The behaviour is also related to 5-HT1 receptors, because the antidepressant acutely increased sexuality while the repeated intervention resulted in minor conflicting effects[43]. Perhaps the whey-induced activity towards females could be related to β-lactoglobulin that forms equimolar complexes with 5-HT and the derivative of which has an affinity for 5-HT receptors[44]. On the other hand, concerning the forced swimming test, it does not appear that the 5-HT

Table 4. Discriminant function coefficients of main behavioural elements that determine the behavioural function*

		Coefficients	
Test	Behaviour	Function 1†	Function 2‡
After 7 d enriched conditions			
Open field	Locomotion through the centre (MD_1)§	−0·968	0·020
	Locomotion around the perimeter (MD_1)	−0·170	0·800
	Contact with the new object (MD_2)§	−0·328	0·556
	Freezing (TF_2)	0·680	−0·778
Light−dark box	Visits into the light compartment (*n*)	0·521	0·399
Forced swimming	Covered distance (mm)	1·616	0·145
	Movement (s)	−1·053	0·649
Group test	Non-aggressive contacts with group members (MD_1)	−0·291	−0·690
	Aggression (MD_1)	1·054	−0·192
	Defence (MD_1)	0·853	0·634
	Latency of non-aggressive contacts (s)	−0·746	−0·419
After 7 d standard conditions			
Open field	Locomotion through the centre (MD_1)	1·169	−0·262
	Locomotion around the perimeter (MD_1)	−0·620	0·417
Elevated-plus maze	Movement in the protected area (*s*)	1·951	−0·845
Forced swimming	Covered distance (mm)	0·440	0·852
	Immobility (MD)	1·883	0·023
Group test	Individual behaviour (RF)	0·872	0·638
	Latency of non-aggressive contacts (s)	−1·144	0·163

MD, medial duration; TF, total frequency; RF, relative frequency.

* Coefficients of behavioural elements are presented to construct linear discriminant functions of behavioural profiles of the dietary groups. The coefficients were calculated by the discriminant function analysis based on individual and social behavioural measurements.

† Function 1 after the enriched conditions = $MD\_1_a \times (−0·968) + MD\_1_b \times (−0·170) + MD\_2_c \times (−0·328) + TF\_2_d \times 0·680 + n_e \times 0·521 + mm_f \times 1·616 + s_g \times (−1·053) + MD_h \times (−0·291) + MD_i \times 1·054 + MD_j \times 0·853 + s_k \times (−0·746)$. Function 1 after the standard conditions = $MD\_1_l \times 1·169 + MD\_1m \times (−0·620) + s_n \times 1·951 + mm_o \times 0·440 + MD_p \times 1·883 + RF_q \times 0·872 + s_r \times (−1·144)$.

‡ Function 2 after the enriched conditions = $MD\_1_a \times 0·20 + MD\_1_b \times 0·800 + MD\_2_c \times 0·556 + TF\_2_d \times (−0·778) + n_e \times 0·399 + mm_f \times 0·145 + s_g \times 0·649 + MD_h \times (−0·690) + MD_i \times (−0·192) + MD_j \times 0·634 + s_k \times (−0·419)$. Function 2 after the standard conditions = $MD\_1_l \times (−0·262) + MD\_1m \times 0·417 + s_n \times (−0·845) + mm_o \times 0·852 + MD_p \times 0·23 + RF_q \times 0·638 + s_r \times 0·163$.

§ 1 and 2, the first and second 3 min intervals that were specified for the open-field test where the observations were done (1) before and (2) after the installation of the new object.

system was activated. It suggests that the increased sexuality may be based on dopamine pathways[39].

The results concerning individual behaviour appeared ambiguous from test to test, although the effects of diets were expected to show parallel results in different tests[25,26]. After the enriched conditions, α-lac-fed mice were more anxious than casein-fed ones in the light−dark box test, while no differences between them were found in the open-field test. The results are in line with an earlier study: animals receiving α-lac for nearly a week showed no differences in open-field behaviour compared with casein-fed ones[4].

However, under the standard conditions, α-lac-fed mice demonstrated less anxiety than casein-fed mice in the open-field test and less anxiety than their peers under the enriched conditions in the light−dark box test. The open-field test, as a more provocative test, produced more diet-specific differences than the light−dark box test. It supports the suggestion that a much more stressful situation or subjects more vulnerable to stress[45] are necessary to indicate the mood-improving effects of the proteins in the post-feeding period. Such a conclusion was made in a human study in which an α-lac diet was ineffective to produce changes in the emotional state of healthy women[9].

The elevated-plus-maze test revealed that α-lac- and whey-fed mice were more mobile than the casein group in the enriched conditions. A similar behavioural activity was shown in the animals exposed to a single meal of an α-lac diet that visited the closed arms as well as the open arms

more often than the animals receiving casein[4]. The increased mobility was found only in the protective area of the maze that is mostly associated with dopamine-induced locomotion[38].

Depression-like status demonstrated by immobility was independent of the diets in the enriched conditions. In the standard conditions, whey-fed mice ceased to swim quicker than casein- and α-lac-fed mice. However, all the animals under the standard conditions demonstrated a delay in immobility and swam longer than their peers in the enriched conditions. This is because the behaviour of whey-fed mice cannot be considered as a real depression-like state or worsened by the diet.

On the whole, all the animals under the enriched conditions appeared to be more anxious in the light−dark box test and displayed more depression-like behaviour in the force swimming test than mice in the standard conditions. Also, α-lac- and whey-fed mice in the standard conditions showed less fear towards the new object in the open-field test than their peers under the enriched conditions. Perhaps the short-term deprivation of the diet and the running wheel was not so dangerous but potentiated a searching activity. Mice that were intermittently withdrawn from food for a short time exhibited less anxiety and depressive behaviour than those regularly receiving food[18]. It seems that the short-term deprivation of a palatable food or a rewarding item might increase the general searching activity (for the reward).

The anxiolytic-like activity of α-lac may be related to the increased level of brain 5-HT that is typical for α-lac-containing

diets[6,8]. In an earlier study, such increased extracellular 5-HT level correlated with the behaviour of rats, which preferred a lighted compartment in the light–dark box test and demonstrated less immobility in the forced swimming test when compared with other animals whose 5-HT level was not changed[46]. Regarding the brain pathways behind the behaviour followed by diets, it is difficult to explain the behaviour profiles only through the 5-HT activity. Thus, in one study[47], β-lactoglobulin, a main component of the whey diet, has been shown to exhibit dopamine-dependent anxiolytic activity in the elevated-plus-maze test. It is possible that a delay in aggression observed in whey-fed mice after the standard conditions might be related to the functioning of the dopamine system. As mentioned previously, the activation of the dopamine system might also be responsible for the increased sexuality of whey-fed mice[48].

In conclusion, all the diet groups demonstrated different behavioural profiles after a prolonged diet intervention. Thus, whey improved sociability while α-lac exhibited anxiolytic and antidepressive activity. The mood-improving properties might be regarded as the behavioural profit of protein feeding that most probably correlates with different brain pathways, which need to be specified in future studies.

Acknowledgements

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