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Casein-based bioactives: how much is too much? Using consumer rejection thresholds to add novel bioactive compounds into model milk-based beverages

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Highlights:

- Bioactive casein hydrolysate (NaCaHs) have potential for use in functional foods.
- Peptide molecular weight influence the bitterness and bioactivity in NaCaHs.
- Rejection threshold models applied to NaCaHs with different bitter profiles.
- Consumer rejection thresholds for the NaCaHs is influenced by the food matrix.

Abstract: This study aimed to identify the amount of crude casein-hydrolysate (HMW) and a low molecular weight sub-fraction (LMW) thereof that could be incorporated into strawberry- and vanilla-flavoured beverages before the bitterness/taste became objectionable to panellists. The beverages were spiked with increasing amounts of hydrolysate and a 2-alternative forced choice (2-AFC) design was employed to determine rejection thresholds (RjT). Results showed a higher amount of HMW, than LMW, could be incorporated into the beverages before the taste became objectionable and the type of flavouring did not have a significant effect on RjT. Following the 2-AFC, panellists rated the bitterness of the hydrolysates (in water) on a general Labelled Magnitude Scale (gLMS). Results showed no significant differences between the bitterness perception of the HMW and LMW. However, there was considerable variation in the panellists' perception of bitterness, suggesting possible evidence for segmentation. Using this rationale, each panel was segmented into two groups: those who rated the bitterness of the hydrolysate samples as ≤ 20 on the gLMS and, those who rated the bitterness as ≥ 75 on the gLMS, and RjT were examined within them. Although a trend was seen for those most sensitive to bitterness in water, to have a lower acceptance for the level in the beverages, the RjT of the segmented groups were not significantly different from each other. Evaluation of hydrolysates in water does therefore not appear to be suitable for predicting consumer acceptance of hydrolysates in food matrices; highlighting the importance of testing food ingredients in the final food product.

Key words: hydrolysate, bitterness, rejection threshold, consumer

1. Introduction

The ghrelin (GHS-R1a) receptor has recently emerged as a novel therapeutic target to stimulate food intake, with potential to benefit those who suffer a loss of appetite from old age and conditions such as cachexia and malnutrition (Howick et al., 2017). Previous work from our group suggests the presence of ghrelin-stimulatory bioactivity in certain sodium caseinate hydrolysates (NaCaHs) and a possible association between such activity and bitterness (Murray et al., unpublished data). However, there are a number of challenges which affects the commercialisation of these NaCaHs; one of which is their taste. NaCaHs are associated with an intense bitter taste (Newman et al., 2014; Murray et al., 2017) which would negatively affect consumption in individuals who already suffer from reduced appetite.

The molecular weight profiles of peptides present in NaCaHs can influence bitterness and bioactivity of the NaCaH. Increased bitterness and bioactivity were observed in low molecular weight and hydrophobic sub-fractions of a NaCaH (Murray et al., 2018). Although the initial intention was to identify non-bioactive, bitter fractions that could be removed to improve the sensory characteristics of the NaCaH as a potential functional ingredient, this finding suggested that removing bitterness would also negatively affect the NaCaH bioactivity. Rather than selectively removing bitter fractions (containing low molecular weight peptides), these particular NaCaHs may benefit from targeted masking strategies to improve their sensory profiles whilst maintaining the functionality of the final product.

Some studies have investigated the masking effect of bitter inhibiting agents for bitterness reduction in dairy protein hydrolysates (DPHs) (Leksrisompong et al., 2012; Newman et al., 2015). Vanilla flavouring, in combination with 0.017–0.03% sucralose, was deemed effective in bitterness reduction in DPHs. Although this work gives insight into flavours, that work well for bitterness masking in this category of ingredient, the level at which these functional

NaCaHs can be incorporated into beverages while remaining acceptable to the consumer is still unknown.

Rejection threshold methods offer a way to determine the point at which certain off-tastes become objectionable in a range of foodstuffs. Prescott et al. (2005) used this technique to model the acceptable level of cork taint in white wine, while more recently the method has been used to examine the addition of sucrose octaacetate (as a model bitter compound) to chocolate milk (Harwood et al., 2012a), solid chocolate (Harwood et al., 2012b) and ice-cream (Harwood et al., 2013). NaCaHs are however, much more complex than sucrose octaacetate (a single bitter compound) as bitterness is not the only taste attribute present which may affect rejection. Thus, it is important to test NaCaHs in real life applications. However, taste perception varies amongst individuals and this is particularly true for bitterness perception, partly due to wide genetic variation in bitter taste receptors (Feeney, 2011). In order to understand the role that variation plays in acceptance, some studies have segmented rejection threshold data based on the panellists' hedonic response to certain foods, such as dark chocolate, to better interpret and understand the results (Harwood et al., 2012a & b). Estimating rejection thresholds could prove to be an effective tool for determining this acceptable limit of NaCaHs incorporated into food products. To our knowledge, there are no published studies on the rejection thresholds of NaCaHs spiked into a food product.

There were two objectives in this study. The first objective was to determine the group rejection thresholds of a range of concentrations of NaCaH samples incorporated into two differently flavoured (vanilla and strawberry) milk beverages, using a series of 2-alternative forced choice (AFC) tests. The hydrolysate samples differed in terms of bitterness and molecular weight profiles: a crude NaCaH, with a higher molecular weight profile (abbreviated as HMW) and a lower molecular weight (<1-kDa permeate) sub-fraction (abbreviated as LMW). The study sought to investigate if flavouring had an effect on the rejection of both

hydrolysate samples and if so, did one flavour (vanilla or strawberry) perform better than the other in masking the bitterness/undesirable attributes of the hydrolysate samples. The second objective was to determine if there were differences in bitterness perception of the hydrolysate samples, within the consumer groups, and how this would affect rejection thresholds of the hydrolysate samples spiked into the flavoured milk drinks. In this study, two approaches were employed to determine the most appropriate approach for determining consumer rejection thresholds: a graphical approach and calculation of best estimate threshold (BET). The graphical approach uses a dose response curve to estimate the point at which panellists started to prefer the control over the spiked samples (R_jT_{50}), while BET is based on the individual geometric means of the panel.

2. Materials and Methods

2.1. Materials

Ultra-high temperature (UHT) milk was gifted by Glanbia Ingredients Ireland. The vanilla (product code: 2SX-87076) and strawberry (product code: 2SX-74444) flavourings were donated by Synergy Flavours Ltd., UK. Sucralose was obtained from Camida, Tipperary, Ireland. The hydrolysate samples (HMW and LMW) were manufactured by our research partners at Teagasc at Moorepark Technology Ltd (Moorepark, Fermoy, Co. Cork, Ireland). All sensory analysis standards were food grade: sucrose, tannic acid, sodium chloride, citric acid, caffeine (Sigma-Aldrich, Ireland) and ferrous sulphate in water (Spatone Ltd, UK).

2.2. Sample preparation

Vanilla and strawberry flavoured milk-based beverages were chosen as the model matrices following advice from industry collaborators, and were spiked with varying concentrations of a hydrolysate sample (HMW and LMW). The flavoured milk drinks were prepared by adding 0.017% (w/v) sucralose (10% sucrose equivalent) and 0.2% (v/v) flavouring (strawberry or

vanilla) to UHT whole milk. The flavoured milk drinks were then spiked with varying concentrations of hydrolysate sample: 0.25, 0.5, 1, 1.5, 2 and 2.5% (w/w); these concentrations were selected based on the outcome of a focus group (n=9). The spiked milk drinks were mixed on a stir-plate for 30 min at room temperature and then stored at 4 °C overnight. For both the 2-AFC testing and the gLMS ratings, samples (10 mL) were served chilled to panellists in clear plastic 30-mL cups (Fast Plast A/S, Denmark) coded with random three-digit numbers.

2.3. Participants

This study was approved by the University College Dublin Human Ethics Committee (Application LS-E-14-90) and all panellists gave informed consent to take part in the study. Four consumer panels were recruited by the UCD Institute of Food and Health, to participate in the study. Eighty-five participants were recruited to each consumer panel, but as not all demographic information was available, some participants were removed, and so each panel then consisted of 83–85 individuals, who were reportedly healthy and non-smokers. The gender and mean age of panellists are reported in Table 1.

2.4. Sensory Testing Procedures

2.4.1. Rejection threshold testing

To determine the rejection threshold of the HMW and the LMW spiked at different concentrations, in vanilla and strawberry flavoured milk drinks, a 2-AFC design was employed. Tests were conducted in the sensory evaluation booths in UCD's Institute of Food and Health. The panellists were served 6 pairs of samples (each pair comprised of a 'blank'/control sample and a 'spiked' sample). The pairs were presented to panellists in order of ascending hydrolysate concentrations and re-tasting of samples (within a pair) was allowed. Redjade software was employed for sample randomisation (in a balanced block design) and data collection. Panellists were asked to indicate the sample in the pair which they preferred by clicking the appropriate

blinding code on the computer screen in front of them. A 'no preference' option was not provided. When evaluating the samples, within each pair, panellists were requested not to rinse their mouth with water but, during a compulsory 30 s break in between each sample pair, panellists were requested to thoroughly rinse their mouth with bottled drinking water.

2.4.2. Taste attribute ratings

After performing the 2-AFC, panellists were asked to taste and rate the bitterness of the hydrolysate sample (either the HMW or the LMW) in water, at a concentration of 2.5% (w/w), on a gLMS. Prior to performing the bitterness ratings, the consumer panel were familiarised with the gLMS. In order for panellists to distinguish between the basic tastes and recognise bitterness, the panel were also familiarised with the following standards dissolved in bottled drinking water: bitter, 0.14% (w/w) caffeine; sour, 0.1% (w/w) citric acid; salty, 0.5% (w/w) sodium chloride; astringent, 0.1% (w/w) tannic acid and metallic, ferrous sulphate solution (Spatone UK), diluted 1:1 ratio with water, to give a final Fe^{2+} concentration of 12.5 mg/100mL (Hayes et al., 2013).

2.5. Statistical analyses

The group rejection threshold (RjT_{50}) was calculated, as per Harwood et al., (2012a), to determine the overall rejected concentration of each hydrolysate sample spiked into both flavoured milk drinks. The proportion of responses indicating a preference for the control were plotted against the concentration of hydrolysate sample spiked into the milk drinks. The points were then fitted to a curve, using the sigmoid variable slope dose-response function with four parameters as described by Harwood et al. (2012a). In order to construct these curves, the rejection functions were fitted directly into GraphPad Prism 7.03 (GraphPad Software, USA), which also directly calculated the RjT_{50} values. The RjT_{50} value is the point at which the panel exceeds the chance preference by 50%, as determined by Abbott's formula (Lawless, 2010). In

a 2-AFC, since there is a 50:50 possibility of participants preferring the control over the spiked flavoured milk drinks, the chance-corrected R_jT_{50} value is 75%. The Hill equation automatically calculates this chance corrected value within the GraphPad Prism software. A number of comparisons were made in order to investigate the effect of flavouring (strawberry and vanilla) and/or the hydrolysate sample (HMW and LMW) on the group rejection threshold. The proportions of those preferring the control verses the spiked beverages were compared in each of the four panels, using 2 x 2 contingency tables and Fisher's exact test (2-tailed) at each concentration.

Additionally, based on the frequency of panellists' bitterness ratings of the hydrolysate sample in water on the gLMS, the 2-AFC data was segmented and rejection thresholds were determined for two groups within each of the four consumer panels; the first group were individuals who rated the bitterness intensity of the hydrolysate sample (2.5% (w/w) concentration in water) as ≥ 75 on the gLMS and the second group were those who rated the bitterness intensity as ≤ 20 on the gLMS. GraphPad Prism was employed to construct the rejection threshold curves of the segmented groups by fitting the data twice into software. The proportions of those preferring the control versus the spiked beverages between the two distinct, segmented groups, in each of the four panels, were compared using 2 x 2 contingency tables and Fisher's exact test (1-tailed) at each concentration.

In addition to calculating the R_jT_{50} values, individual BETs were calculated to more accurately determine the acceptable level of hydrolysate sample that could be incorporated into the flavoured drinks. A BET is the geometric mean of the first concentration level of a compound that is detected correctly, with all higher concentrations correct, and the next lower level concentration of a compound that was answered incorrectly (Lawless, 2010; Lawless & Heymann, 2010). However, in this present study, calculation of the BET was adapted slightly and was defined as the geometric mean of the first concentration of hydrolysate sample at

which, the participant chose the control as the preferred sample (and subsequently chose the control samples for the remainder of the test) and the next lowest concentration of hydrolysate where a participant chose the hydrolysate spiked sample over the control. The group BET was calculated as described by Lawless (2010). Briefly, the individual BET values were converted to Log_{10} values and the mean was calculated, which was then subsequently converted to the antilog to determine the group BET value, or the concentration at which the group started to prefer the control over the spiked samples. For statistical analysis of the BET, the individual $\text{Log}_{10}(\text{BET})$ values were input into SPSS. Again, a number of comparisons were made in order to investigate the effect of flavouring (strawberry and vanilla) and/or the hydrolysate sample (HMW and LMW) on the group BET; these comparisons were made using a paired (2-tailed) t-test. The BETs between the two distinct segmented groups were compared using the Mann Whitney test as this data was not normally distributed.

3. Results

3.1. Consumer/Group rejection

Group rejection threshold is defined as the concentration at which the participants preferred the 'control' over the spiked samples (Prescott et al., 2005; Harwood et al., 2012a). In this study, two approaches were employed to determine group rejection thresholds: a graphical approach and calculation of BET.

3.1.1. Determining consumer/group RjT_{50} using the graphical approach

To determine RjT_{50} , using the graphical approach, the proportion of responses indicating preference for the 'control' were plotted against the concentration of hydrolysate spiked in the flavoured milk drinks (not shown). A curve was then fitted through the points, using a sigmoid fit. However, due to the lack of points near the chance (0.5) for the original sigmoid fit, of the flavoured milk drinks spiked with the LMW, the RjT_{50} values of these samples were

ambiguous. Therefore, it was decided to constrain two of the four parameters (the minimum and maximum values) in the Hill equation to their theoretically indicated values of 0.5 and 1, respectively, and refit the curve (Figure 1). In order to best compare R_jT_{50} between the consumer panels, it was decided to constrain all data as described above.

Overall, as the concentration of hydrolysate sample spiked into the flavoured milk drinks increased, the more the participants preferred the blank/control sample (Figure 1). None of the consumer panels completely rejected – i.e. rejection by 100% of panellists – the flavoured milk drinks spiked with either of the hydrolysate samples, even at the highest level of addition. The proportion of participants preferring the control over the HMW-spiked beverages were 98% and 94% for Consumer Panel 1 and 2, respectively, while 96% and 99% of participants from Consumer Panel 3 and 4, respectively, preferred the control over the LMW-spiked beverages.

The proportions of those preferring control vs spiked were compared in each of the four panels, using 2 x 2 contingency tables and Fisher's exact test (2-tailed) at each concentration. The beverages spiked with HMW had higher R_jT_{50} values (0.79 and 0.77% (w/w) in strawberry and vanilla beverages, respectively) than those spiked with LMW (0.51 and 0.46% (w/w) in strawberry and vanilla beverages, respectively) (Figure 1). Although the strawberry-flavoured beverages (spiked with the hydrolysate samples) had higher R_jT_{50} values compared to vanilla-flavoured beverages, the type of flavouring did not have a significant effect on the overall group rejection thresholds of the HMW and LMW ($p > 0.05$; see Table 2 for *p-values*). However, when the HMW versus the LMW were compared, the results are suggestive of the HMW being preferred over the LMW, and this effect was seen more strongly (and significantly) in the vanilla flavour group (see Table 3 for *p-values*).

3.1.2. Determining consumer/group rejection with BET

The BETs of the individual panellists, within each of the consumer panels, were also calculated and from these values, group BETs were calculated. The group BETs were lower than the group RjT_{50} values, as determined from the dose response curves (Figure 2). Calculating BET may, therefore, be a more conservative approach in determining group rejection thresholds than calculating RjT_{50} values from a dose response curve. Nevertheless, both forms of analysis are deemed appropriate to determine group thresholds (Lawless & Heymann, 2010).

A paired (2-tailed) t test was employed to determine if the mean difference between the BETs of the consumer panels was significantly different. Normality of the groups was assessed by visual inspection of a Normal Q-Q plot. The difference scores between the groups were normally distributed. The flavoured milk drinks spiked with HMW had significantly higher BET values than those spiked with LMW ($p = 0.019$ for the strawberry-flavoured beverages; $p = 0.000$ for the vanilla-flavoured beverages), indicating that the LMW-spiked beverages were less desirable than the HMW-spiked beverages (Figure 2). The strawberry-flavoured beverages (spiked with hydrolysate) had higher BET values than the vanilla-flavoured beverages (Figure 2), however, the type of flavouring did not have a significant effect on the group BETs of the hydrolysate samples ($p = 0.981$ for the strawberry-flavoured beverages; $p = 0.224$ for the vanilla-flavoured beverages). Although the type of flavouring did not have a significant effect on the rejection thresholds of the hydrolysate samples, the higher RjT_{50} and BET values of the strawberry drinks spiked with hydrolysate (Figure 2) may, to some extent, indicate that strawberry flavouring, has slightly better bitterness masking abilities than the vanilla flavouring.

3.2. Bitterness Intensity Ratings

At the end of the 2-AFC tests, participants were asked to evaluate the bitterness of a single hydrolysate sample (either HMW or LMW) at a concentration of 2.5% (w/w) in water on a gLMS. The mean bitterness scores of the HMW were 49.5 ± 23.5 and 49.7 ± 24.5 , for consumer panels 1 and 2, respectively. The mean bitterness scores of the LMW were 47.0 ± 27.2 and 49.3 ± 25.4 for consumer panels 3 and 4, respectively. Results showed the mean bitterness intensities of the HMW and LMW were not significantly different ($p = 0.538$, bitterness values between panels 1 and 3; $p = 0.931$, bitterness values between panels 2 and 4). The large standard deviations of the ratings indicates a wide variation in bitterness perception of both samples (Figure 3). A large number of panellists rated the bitterness of the hydrolysate samples in the 50–55 range on the gLMS, equating to approximately ‘strong’ taste intensity. However, some participants rated the bitter intensity of the hydrolysate as less than 17 (moderate) on the gLMS, with some even giving the bitterness of the sample a rating of just 1 on the scale. In contrast, other participants rated the bitterness of the samples as high as 100 on the scale. As a result, potential segmentation of acceptability based on bitterness perception was explored. Based on the frequency plots, the consumer panels were categorised into two extreme groups: 1) those who rated the bitterness intensity of 2.5% (w/w) hydrolysate sample in water as ≤ 20 on the gLMS, and 2) those who rated the bitterness intensity as ≥ 75 on the gLMS. These cut-off points were based on visual inspection of the frequency plots (Figure 3) and finding the best cut-off point to best suit all consumer panels.

3.2.1. Determining R_jT_{50} of the segmented groups using the graphical approach

The rejection thresholds of the segmented groups were constructed again using a sigmoid fit function. However, the lack of points near the chance (0.5) resulted in ambiguous R_jT_{50} values and thus, it was necessary to constrain the data and re-fit the curves as previously described (3.1.1) (Figure 4). The proportions of those preferring the control versus the spiked beverages

between the two distinct, segmented groups, in each of the four panels, were compared using 2 x 2 contingency tables and Fisher's exact test (1-tailed) at each concentration. The panellists who rated the bitterness intensity of the hydrolysate samples as ≤ 20 on the gLMS had higher R_jT_{50} values than those who rated the bitterness of the sample as ≥ 75 on the gLMS, however, this difference was not significant (see Table 4 for *p-values* for the comparison of the segmented groups of each consumer panel for each level of hydrolysate concentration).

3.2.2. Determining rejection threshold of the segmented groups with BET

Individual BETs were also calculated for each of the segmented groups and, from these values, the mean BETs of the segmented groups were calculated. The majority of the BET values for the segmented groups were not normally distributed, as assessed by the Shapiro-Wilk's normality ($p > 0.05$). The data was positively skewed, as a high number of panellists within each of the segmented groups preferred the control samples over the spiked samples. Therefore, the Mann Whitney test was used to determine the significance of any differences between the groups. The group of individuals who rated the bitterness of the hydrolysate as ≤ 20 on the gLMS had higher BETs than those who rated the bitterness of the samples as ≥ 75 on the scale, however, again, no significant differences were observed between the segmented groups ($p > 0.05$) (Table 5).

4. Discussion

In this study, two approaches were employed to determine group rejection thresholds: a graphical approach and calculation of BET. The graphical approach uses a dose response curve to estimate the point at which panellists started to prefer the control over the spiked samples (R_jT_{50}). Forced choice procedures (such as the 2-AFC) tend to overestimate the group threshold as the dose response curve is generated by plotting proportion of responses preferring the control at each hydrolysate concentration spiked into the flavoured milk. In contrast, BET is

based on the individual geometric mean of each consumer in the panel and thus, interpolation of BET may provide some protection against this overestimation (Lawless & Heymann, 2010) as in general, BETs were lower than the R_jT_{50} values (as determined from the dose response curves).

While both forms of analysis are deemed appropriate to determine group thresholds (Lawless & Heymann, 2010), BET is typically used for determining detection thresholds rather than consumer rejection thresholds. A potential limitation with BET is that there is an assumption that the concentration of bitterness should reach a point where it becomes objectionable, this however may not be the case for everyone as some panellists may prefer increased bitterness. Rejection threshold testing is still a relatively new concept/development and therefore, more research is needed to better understand the relative merits and demerits between using BET and R_jT_{50} for determining rejection thresholds. Thus, it may be beneficial for researchers to use both approaches in determining consumer rejection thresholds.

The proportions of those preferring control vs spiked in each of the four panels (Figure 1), were compared using 2 x 2 contingency tables and Fisher's exact test (2-tailed) at each concentration. Examining them in this way indicated that there is no difference between the strawberry and vanilla flavours in the level of rejection for HMW or for the LMW when considered alone (Table 2). However, when the HMW versus the LMW themselves were compared, the results were suggestive of the HMW being preferred over the LMW, and this effect was seen more strongly in the vanilla flavour group (Table 3). The strawberry-flavoured beverages had higher BET values than the vanilla-flavoured beverages (Figure 2), however, the type of flavouring did not significantly affect the consumer/group BETs of the hydrolysate samples ($p = 0.981$ for the strawberry-flavoured beverages; $p = 0.224$ for the vanilla-flavoured beverages). Although the type of flavouring did not have a significant effect on the rejection

thresholds of the hydrolysate samples, the higher R_jT_{50} and BET values of the strawberry-flavoured beverages spiked with hydrolysate may, to some extent, indicate slightly better masking ability of the strawberry flavouring compared to the vanilla flavouring. Previous studies investigated the masking ability of vanilla flavouring on DPH bitterness (Leksrisompong et al., 2012; Newman et al., 2015).

The bitterness ratings of the hydrolysate samples in water show no significant difference in bitterness between the HMW and the LMW. This was surprising, considering that hydrolysate bitterness is associated with an increased concentration of low molecular weight peptides (Leksrisompong et al., 2010; Murray et al., 2017). However, the large standard deviations of the bitterness ratings of the hydrolysate samples in water highlighted the wide variation in how consumers perceived the bitterness of the HMW and LMW. Therefore, looking at the mean bitterness ratings may not be the best approach in understanding consumers' perception of bitterness in the HMW and LMW since it may overlook issues like variation in the sensitivity to bitterness among different consumer groups. Interestingly, while there were no significant differences between the bitterness intensities of the hydrolysate samples in water, the flavoured milk drinks, spiked with HMW, had significantly higher BET values than those spiked with LMW ($p = 0.019$ for the strawberry-flavoured beverages; $p = 0.000$ for the vanilla-flavoured beverages). This indicates that the LMW-spiked beverages were less desirable than the HMW-spiked beverages perhaps suggesting that more HMW (than LMW) can be incorporated into the flavoured milk drinks, before the taste becomes objectionable to the panellists. Therefore, the matrix into which hydrolysates are incorporated into (e.g. water or flavoured milk) may have an important effect on rejection. Previous work from our group demonstrated that the presence of sucralose can alter the perceived flavour profile of NaCaHs, with an increase in 'cheesiness' being detected, as the sucralose concentration increased from 0.0025–0.01% (w/w) (Hutchings et al., 2017). This highlights the importance of evaluating the consumer

acceptability/taste attributes of NaCaHs in real food products, due to possible interaction of the NaCaH with the food matrix.

The consumer panels were segmented into two extreme groups, based on their bitterness perception of the hydrolysate sample in water. The group of individuals who rated the bitterness of the hydrolysate as ≤ 20 on the gLMS had higher BETs than those who rated the bitterness of the samples as ≥ 75 on the scale. However, no significant differences were observed between these segmented groups ($p > 0.05$) (Table 5). A number of reasons may account for this lack of significance. The numbers of panellists within the segmented groups are quite low and therefore a larger sample size may result in significance. Bitterness is also not the only unfavourable taste attribute present in NaCaHs (Newman et al., 2014). Other side tastes present in the NaCaHs may influence rejection of the NaCaHs when incorporated into a food matrix, such as flavoured milk. Although the individuals who rated the bitterness of the hydrolysate samples (in water) as ≤ 20 on the gLMS may be more tolerant due to a lowered perception of hydrolysate bitterness, this may not necessarily mean that they would prefer hydrolysate-spiked samples over the control/blank samples. It seems tolerance for hydrolysate bitterness does not predict liking and/or rejection of hydrolysates incorporated into a food matrix, such as flavoured milk. This highlights the complexity of determining consumer acceptance of hydrolysates and the need to evaluate the sensory profiles of hydrolysates incorporated into food matrices.

5. Conclusion

NaCaHs have great potential to positively influence health but their intense bitter taste is a major hindrance for their commercialisation. The findings from this study highlighted the serious challenge of incorporating hydrolysates into food products, as only limited amounts ($< 1\%$ w/w) of hydrolysate could be incorporated into flavoured milk beverages before the

undesirable taste of the hydrolysates became objectionable. A significantly higher amount of HMW, than LMW, was incorporated into flavoured milk without the taste becoming objectionable. Surprisingly, bitterness ratings of the hydrolysate samples in water showed no significant difference in bitterness between the hydrolysate samples. However, the distribution of the bitter ratings highlighted considerable variation in the consumers' perception of hydrolysate bitterness and thus, segmentation within the consumer panels was investigated. That said, the rejection thresholds of these distinct, segmented groups were not significantly different. This therefore highlights the importance of testing food ingredients in food matrices rather than in water, as no significant difference in bitterness were observed between the HMW and LMW in water, despite the significant difference between the rejection thresholds of these hydrolysate samples incorporated into flavoured milk. Therefore, the evaluation of hydrolysates in water does not appear to be reflective of final consumer acceptance/rejection of hydrolysates once incorporated into a food matrix.

Abbreviations

AFC, alternative forced choice; UHT, ultra-high temperature; gLMS, general Labelled Magnitude Scale; NaCaH, sodium caseinate hydrolysate; HMW; high molecular weight hydrolysate; LMW, low molecular weight hydrolysate; DPH, dairy protein hydrolysate; BET, best estimate threshold

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Figure 1. Proportion of participants preferring the control/uns spiked flavoured milk drinks plotted against the concentration of hydrolysate spiked into the strawberry (A) and vanilla (B) flavoured milk drinks. The curve was fit with a constrained sigmoid fit.

Figure 2. Comparison of the RjT₅₀ (■) and BET (■) values of the hydrolysate samples (HMW and LMW) in the strawberry and vanilla flavoured milk. 95% CI of BETs: 0.49 (0.41, 0.57), 0.35 (0.27, 0.44), 0.49 (0.40, 0.58) and 0.31 (0.25, 0.38).

Figure 3. Frequency distribution of the bitterness intensities of 2.5% (w/w) hydrolysate sample in water as rated on a gLMS; No sensation=0, Weak=6, Moderate=17, Strong=35, Very strong=51 and Strongest Imaginable Sensation of any kind=100. Data was obtained from Consumer Panel 1 (A), Consumer Panel 2 (B), Consumer Panel 3 (C) and Consumer Panel 4 (D).

Figure 4. Proportion of participants preferring the control/uns spiked flavoured milk drink plotted against the concentration of hydrolysate spiked in the flavoured milk drinks. The squares (■) represent the individuals who rated the bitterness of 2.5% (w/w) hydrolysate in water ≥ 75 on the gLMS; the circles (●) represent individuals who rated the bitterness of the hydrolysate in water ≤ 20 on the gLMS. The curve was fit with a constrained sigmoid fit.

Table 1. Consumer panel demographics.

Panel	(Spiked) Milk Drink	Number of consumers	Gender		Mean Age
			Male	Female	
1	(HMW) Strawberry milk	83	27	56	40
2	(HMW) Vanilla milk	85	28	57	44
3	(LMW) Strawberry milk	84	28	56	39
4	(LMW) Vanilla milk	85	32	53	39

Table 2. Comparisons between Groups: Effect of flavour on rejection thresholds

Hydrolysate Concentration	Proportion preferring the control in:		HMW (Strawberry vs. Vanilla)	Proportion preferring the control in:		LMW (Strawberry vs. Vanilla)
	Strawberry beverages	Vanilla beverages		Strawberry beverages	Vanilla beverages	
0.25 %	51 %	56 %	$p = 0.5362$	55 %	52 %	$p = 0.7585$
0.5 %	63 %	65 %	$p = 0.8727$	77 %	81 %	$p = 0.5737$
1 %	82 %	82 %	$p = 1.00$	92 %	94 %	$p = 0.5655$
1.5 %	93 %	89 %	$p = 0.5903$	96 %	98 %	$p = 0.6819$
2 %	94 %	91 %	$p = 0.5657$	94 %	99 %	$p = 0.1172$
2.5 %	99 %	94 %	$p = 0.2105$	96 %	99 %	$p = 0.3675$

*The proportions of those preferring the control verses the spiked beverages were compared in each of the four panels, using 2 x 2 contingency tables and Fisher's exact test (2-tailed) at each concentration.

Table 3. Comparisons between Groups: Effect of hydrolysate on rejection thresholds

Hydrolysate Concentration	Proportion preferring the control in:		Strawberry (HMW vs. LMW)	Proportion preferring the control in:		Vanilla (HMW vs. LMW)
	HMW beverages	LMW beverages		HMW beverages	LMW beverages	
0.25 %	51 %	55 %	$p = 0.5362$	56 %	52 %	$p = 0.6444$
0.5 %	63 %	77 %	$p = 0.0435$	65 %	81 %	$p = 0.0242$
1 %	82 %	92 %	$p = 0.071$	82 %	94 %	$p = 0.0536$
1.5 %	93 %	96 %	$p = 0.3287$	89 %	98 %	$p = 0.057$
2 %	94 %	94 %	$p = 1$	91 %	99 %	$p = 0.0342$
2.5 %	99 %	96 %	$p = 0.621$	94 %	99 %	$p = 0.2104$

*The proportions of those preferring the control verses the spiked beverages were compared in each of the four panels, using 2 x 2 contingency tables and Fisher's exact test (2-tailed) at each concentration.

Table 4. Comparisons between the segmented groups of each consumer panel at each hydrolysate concentration

Hydrolysate concentration	Proportion preferring the control in:		Panel 1	Proportion preferring the control in:		Panel 2	Proportion preferring the control in:		Panel 3	Proportion preferring the control in:		Panel 4
	Segmented group 1	Segmented group 2		Segmented group 1	Segmented group 2		Segmented group 1	Segmented group 2		Segmented group 1	Segmented group 2	
0.25 %	33 %	53 %	$p = 0.3001$	64 %	50 %	$p = .3278$	53 %	44 %	$p = 0.429$	50 %	55 %	$p = 0.524$
0.5 %	78 %	80 %	$p = 0.6403$	57 %	72 %	$p = 0.3023$	71 %	88 %	$p = 0.2247$	79 %	75 %	$p = 0.6516$
1 %	56 %	87 %	$p = 0.113$	79 %	89 %	$p = 0.376$	82 %	94 %	$p = 0.1313$	1 %	95 %	$p = 0.5882$
1.5 %	1 %	87 %	$p = 0.3804$	86 %	94 %	$p = 0.4036$	94 %	1 %	$p = 0.5152$	1 %	1 %	$p = 1$
2 %	89 %	93 %	$p = 0.6196$	79 %	1 %	$p = 0.0734$	94 %	94 %	$p = 0.7424$	1 %	1 %	$p = 1$
2.5 %	1 %	1 %	$p = 1$	93 %	1 %	$p = 0.4375$	94 %	1 %	$p = 0.5152$	93 %	1 %	$p = 0.4118$

*The proportions of those preferring the control versus the spiked beverages were compared in each of the four panels, using 2 x 2 contingency tables and Fisher's exact test (1-tailed) at each concentration.

** Segmented group 1= those who rated the bitterness of the hydrolysate samples as ≤ 20 on the gLMS; Segmented group 2 = those who rated the bitterness as ≥ 75 on the gLMS.

Table 5. Best estimate threshold (BET) of the segmented groups.

Group	<i>n</i>	BET (w/w %)	<i>P</i>-value*
HMW in strawberry milk (Panel 1)	85	0.49	
Bitter Rating ≤ 20	9	0.69	0.3179
Bitter Rating ≥ 75	15	0.43	
HMW in vanilla milk (Panel 2)	85	0.49	
Bitter Rating ≤ 20	14	0.64	0.1484
Bitter Rating ≥ 75	18	0.38	
LMW in strawberry milk (Panel 3)	85	0.35	
Bitter Rating ≤ 20	17	0.46	0.2239
Bitter Rating ≥ 75	16	0.30	
LMW in vanilla milk (Panel 4)	85	0.31	
Bitter Rating ≤ 20	14	0.34	0.6655
Bitter Rating ≥ 75	20	0.29	

*Variation between the BET values of the segmented groups was determined by the Mann Whitney test.

Highlights:

- Bioactive casein hydrolysate (NaCaHs) have potential for use in functional foods.
- Peptide molecular weight influence the bitterness and bioactivity in NaCaHs.
- Rejection threshold models applied to NaCaHs with different bitter profiles.
- Consumer rejection thresholds for the NaCaHs is influenced by the food matrix.