EUROPEAN MEDICINES AGENCY
SCIENCE MEDICINES HEALTH

15 January 2025
EMA/CVMP/375793/2024
Committee for Veterinary Medicinal Products

Scientific advice on MRL classification of chemical-unlike biological substances considered as not requiring an MRL evaluation according to Regulation (EU) 2018/782

1. Introduction

Five substances have previously been considered by CVMP and were included in the list of chemical-unlike biological substances considered as not requiring an MRL evaluation as per Regulation (EU) No. 2018/782, with regard to residues of veterinary medicinal products in foodstuffs of animal origin (EMA/CVMP/572629/2019-Rev.2): bovine casein hydrolysate (bCNH), probiotic components including bacteria and yeasts, recombinant bovine IL-8 (His-tag), stem cells, and varroa destructor calmodulin gene-specific double-stranded interfering RNA EP15 (naked unmodified dsRNA).

Concern has arisen that substances included in the above-mentioned list could be considered 'unauthorised substances' as per the definition provided by Article 2 of Commission Delegated Regulation No. (EU) 2019/2090. In order to address this concern, a modification of the Annex to Commission Regulation (EU) No. 2018/782 is proposed, according to which, in future, substances evaluated according to section I.7 of that annex will either qualify for a "No MRL required" classification in the Table 1 of the annex to Regulation (EU) No. 37/2010, or, if such an entry is not considered appropriate following the evaluation undertaken according to section I.7 of the annex to Commission Regulation (EU) No. 2018/782, an MRL application according to Regulation (EC) No. 470/2009 will be required.

In order to ensure consistency of possible outcomes for all substances considered in line with section I.7 of the annex to Commission Regulation (EU) No. 2018/782, the European Commission requested the Agency, on 13 September 2024, to provide scientific advice confirming that the five substances already included in the list of chemical-unlike biological substances considered as not requiring an MRL evaluation do not pose any risk to public health and that their inclusion in Table 1 of the annex to Regulation (EU) No. 37/2010 with a "No MRL required" classification is appropriate.

CVMP appointed Andrea Golombiewski as rapporteur and Carina Bergman as co-rapporteur on 10 October 2024.

This advice was submitted to the CVMP on 20 November 2024.

The CVMP adopted the advice on 15 January 2025.

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2. Scientific assessment

Bovine casein hydrolysate (bCNH), produced from sodium caseinate hydrolysed with trypsin, heat treated, for intramammary use in cows

Bovine casein hydrolysate (bCNH) was previously evaluated in accordance with the provisions of section I.7 of the annex to Commission Regulation (EU) No. 2018/782. It was concluded that the bCNH assessed represented a chemical-unlike biological substance for which no further MRL assessment was necessary, resulting in inclusion on the respective list of substances with the following entry: "Bovine casein hydrolysate (bCNH), produced from sodium caseinate hydrolysed with trypsin, heat treated, for intramammary use in cows." A report summarising the CVMP's evaluation was published in January 2020 (EMA/2278/2020) and is available here. The CVMP confirms that the conclusions reached in January 2020 remain applicable today.

Moreover, taking into account the criteria set out under section I.2.2 of Annex II to Commission Regulation (EU) No. 2018/782, CVMP considers that a 'No MRL required' classification is appropriate for the following reasons:

- The basis for the manufactured bCNH is bovine sodium caseinate stemming from milk collected for human consumption, and as such represents a biological substance of endogenous origin. Bovine casein is the main structural protein of cow's milk and accounts for approximately 80% of the total milk protein. The manufacturing process of bCNH involves hydrolysation of sodium caseinate by trypsin as well as subsequent heating to inactivate said enzyme.

  Native bCNH acts as part of the internal metabolism process and during evolution/involution processes of the bovine mammary gland. As such, endogenous bCNH is present in milk prior to and during involution of the mammary gland. The ratio of hydrolysed to non-hydrolysed casein, however, is unknown.

  Although the naturally occurring bCNH is assumed to be formed through hydrolysis by plasmin and further proteases as opposed to the digestive enzyme trypsin, it is considered comparable to the manufactured bCNH, since the peptidase activity of plasmin is very similar to that of trypsin. Qualitative and quantitative composition of manufactured and native bCNH was found to be closely similar. Furthermore, the HPLC spectrum of the hydrolysed product was compared to HPLC spectra of functional food and baby foods which consist of or contain bCNH and were not considered significantly different (fractions of sodium caseinate present in bCNH will vary depending on the points of contact of enzyme and protein; consequently, spectra will not be identical).

  In summary, while bCNH in veterinary medicinal products is the result of a manufacturing process, it is reasonable to accept that it has similar properties to a normal constituent of the diet in humans as well as in animals (calves) due to its similarity in composition to native bCNH in milk and its use in functional/baby foods.

- Concerning oral toxicity of manufactured bCNH, casein components are known food allergens, but the enzymatic hydrolysis is likely to reduce the allergenicity compared to native casein/casein hydrolysate. Potentially immunogenic bCNH polypeptides reactions from bCNH treated animals are not considered to present a hazard different from that of native caseins. Due to the restriction to intramammary use, manufactured bCNH will only be found in foods that also contain native milk ingredients, so that no unexpected risk for hypersensitivity reactions arises from manufactured bCNH. With regard to the potential pharmacological activity of bCNH, both hydrolysed and native casein undergo the same enzymatic and non-enzymatic degradation processes in the human digestive tract. While biologically relevant pharmacological effects of small peptides (di-and tripeptides) from casein

precursors in general cannot be ruled out (including antihypertensive and opioid receptor binding effects), the peptide composition resulting from ingestion of bCNH residues in food commodities can be expected to be similar to that of naturally occurring casein/casein hydrolysate from the diet. I.e., if there were any potential pharmacological effects from the ingestion of bCNH, they would be similar to those from naturally occurring casein from food.

- No studies on absorption, distribution, metabolism or excretion of the product in the target animals were available. In a worst-case exposure assessment for consumers based on a total dose of 4800 mg manufactured bCNH (1200 mg per udder quarter), representing the intended dose which could result in respective residues in one milking at a 12-hour milking interval, the treatment-related casein and casein hydrolysate intake would be below 9.2% and 50% of the estimated total daily intake of caseins. This is considered to be within the natural range of milk proteins in milk. Accepting similarity to naturally occurring bovine casein/hydrolysed casein, residues from bCNH resulting from udder treatment are not expected to differ from those resulting from naturally produced casein already present in milk (and in much lower amounts also in edible tissues, as it cannot be excluded that small quantities of casein or bCNH, native or manufactured, enter the systemic circulation in cows with mastitis), also regarding their bioavailability and nutritional-physiological characteristics. In addition, consumers may already be exposed to hydrolysed casein through food supplements, and infants may be exposed through formula milk for babies.

  Overall, exposure to residues of manufactured bCNH from intramammary treatment is expected to have only a minor impact on the overall exposure to bCNH.

Recombinant bovine IL-8 (His-tag) for intrauterine use in cattle at a dose of up to 1,000 µg per animal

Recombinant bovine IL-8 (rbIL-8) was previously evaluated in accordance with the provisions of section I.7 of the annex to Commission Regulation (EU) No. 2018/782. It was concluded that the substance could be added to the respective list of chemical-unlike biological substances considered as not requiring an MRL evaluation with the following entry: "Recombinant bovine IL-8 (His-tag) for intrauterine use in cattle at a dose of up to 1,000 µg per animal." A report summarising the CVMP's evaluation was published in January 2020 (EMA/CVMP/608257/2020) and is available here. The CVMP confirms that the conclusions reached in January 2020 remain applicable today.

Furthermore, reflecting on the criteria stipulated under section I.2.2 of Annex II to Commission Regulation (EU) No. 2018/782, CVMP considers that a 'No MRL required' classification is appropriate for the following reasons:

- Recombinant bIL-8, produced in *E. coli* strain One Shot BL21 Star (DE3) containing the pET28b plasmid, consists of a 79 amino acid variant from the gene sequence of bovine IL-8 (bIL-8) as well as a 40 amino acid peptide leader sequence, which includes a 6-histidine tag utilized for purification purposes. Hence, only part of the peptide is similar to bIL-8.

  While the His-tagged rbIL-8 protein does not represent a normal constituent of the human diet, natural bIL-8 is present in food of animal origin. Bovine IL-8 concentrations in milk range between 0 and 6.17 ng/ml.

- Concerning its pharmacological effect, as a chemoattractant IL-8 binds to high-affinity receptors present on the surface of neutrophilic granulocytes. This biologically relevant activity, attracting human neutrophils, has a similar potency in rbIL-8, recombinant human IL-8 (rhIL-8) and native bIL-8 *in vitro*. Furthermore, rbIL-8 was shown to activate human CXCR1/2 receptors (hCXCR1/2) to a lesser degree than rhIL-8 or bIL-8.

In addition, the potential for any pharmacological effect of orally ingested rbIL-8 residues is limited by its extensive degradation in the adult human digestive tract, so that relevant local effects in the human gut are considered unlikely.

In human infants, local effects of rbIL-8 in the gut are deemed possible based on literature reports of *in vitro* studies and decreased digestion in infants as compared to adults, which is due to the higher gastric pH value in infants. With regard to the natural exposure of infants to hIL-8 through human breast milk (which contains up to 13 ng hIL-8/ml) and the lower activity of rbIL-8 on hCXCR1/2 when compared to bIL-8, the risk for infants of local effects in the gastrointestinal tract from consuming rbIL-8 can nevertheless be considered low.

- Based on comparison of the sequence of rbIL-8 to known allergen databases, rbIL-8 is not likely to exhibit allergenic cross-reactivity in comparison to known allergens, and the risk for immunogenicity is similar to that of consuming bovine milk that does not contain rbIL-8.
- Following intrauterine treatment at a total dose of 500 µg/animal, rbIL-8 was detected in milk samples of only 1 out of 24 cows at concentrations corresponding to endogenous levels of bIL-8 (all other samples were below the lower LOQ of 3.13 ng/ml). Concentrations of natural bIL-8 levels in meat and offal, or residue concentrations of rbIL-8 in edible tissues after intrauterine application are not available.

  However, the substance is effectively digested in the stomach and intestine, with systemic exposure through rbIL-8 being estimated at < 1% of human endogenous IL-8 levels in adults. Low oral bioavailability of rbIL-8 was also concluded from studies in rats.

  In conclusion, worst-case estimation of possible combined exposure (rbIL8 + bIL8) shows that there is no increased risk for the consumer following intrauterine treatment of cows with rbIL-8 up to a dose of 1,000 µg bovine IL-8.

Varroa destructor calmodulin gene-specific double-stranded interfering RNA EP15 (naked unmodified dsRNA)

Varroa destructor calmodulin gene-specific double-stranded interfering RNA EP15 (naked unmodified dsRNA) was previously evaluated in accordance with the provisions of section I.7 of the annex to Commission Regulation (EU) No. 2018/782 and concluded that it is a chemical-unlike biological substance and no further MRL assessment was necessary for Varroa destructor calmodulin gene-specific double-stranded interfering RNA EP15.

A report summarising the CVMP's evaluation was published in November 2023 (EMA/CVMP/449785/2023) and is available here. The CVMP confirms that the conclusions reached in November 2023 remain applicable today.

Moreover, CVMP considers that a 'No MRL required' classification is appropriate for the following reasons:

- Varroa destructor calmodulin gene-specific double-stranded interfering RNA EP15 is not endogenous in humans. Instead, it is an dsRNA, that is cleaved into short duplex sequences of 21 nucleotides (siRNAs) in the cytoplasm of target cells via cell own Dicer and Dicer-like proteins. While this specific dsRNA or the resulting cleaved siRNAs are not part of the human diet, in general, different types of unmodified RNAs are normal constituents of the diet of humans and animals. As dsRNA or siRNA are not modified, they can be seen as similar to common dietary RNAs, which are essential nutrients, as will their fate in the consumer (digestion).
- For (ds)RNA and/or siRNAs to have pharmacological activity, they are required to enter cells, as the function is gene silencing by binding of siRNA to intracellular RNA. Orally ingested dsRNA/siRNA would

most likely encounter several physical and biochemical barriers (e.g. nucleases in the saliva and gastrointestinal tract, acidic conditions in the stomach, multi membrane barriers including the gastrointestinal tract), preventing them from reaching relevant systemic availability in the cytoplasm of mammalian cells.

- Varroa destructor calmodulin gene-specific double-stranded interfering RNA EP15 is designed to specifically interfere with the transcription of the Varroa destructor calmodulin gene of Varroa mites. There is no complete alignment to human transcriptome and only three potential partial alignment off-target sequences were identified in bioinformatic analysis. One potential off-target partial alignment was identified for which the likelihood of binding to the human transcriptome was not low. However, as laid out above, it can be expected that the one potential off-target partial alignment would likely not reach cells in sufficient quantities to mediate biological effects. Based on these considerations, the Varroa destructor calmodulin gene-specific double-stranded interfering RNA EP15 is not expected to have pharmacological activity considered to be biologically relevant in humans.
- The submitted toxicity studies showed low toxicity but are only indicative and no information about the detectable residues in food derived from treated animals is available, however, this is considered acceptable due to the expected low cellular bioavailability and low likelihood of pharmacological activity of the Varroa destructor calmodulin gene-specific double-stranded interfering RNA EP15 in mammals.

Stem cells

Stem cells were considered by CVMP in December 2012, well before Regulation (EU) No. 2018/782 and the related list of chemical-unlike biological substances considered as not requiring an MRL evaluation as per Regulation (EU) No. 2018/782 existed. The CVMP recognised that a requirement for an MRL application for stem cells could not be justified on scientific grounds and there was therefore agreement to include stem cells in the list of substances considered as not falling within the scope of Regulation (EC) No. 470/2009, with regard to residues of veterinary medicinal products in foodstuffs of animal origin (EMA/CVMP/519714/2009), under the heading of Biologically active constituents. With the creation of the list of chemical-unlike biological substances considered as not requiring an MRL evaluation as per regulation (EU) No. 2018/782, it was considered appropriate to move stem cells to that list. The CVMP remains of the opinion that no further MRL assessment is necessary for stem cells. However, no report summarising the CVMP's evaluation was published at that time.

Therefore, shortly, the CVMP considered that:

Stem cells are cells with the ability to divide for indefinite periods in culture and to give rise to specialized cells. Stem cells are distinguished from other cell types by two important characteristics. First, they are unspecialized cells capable of renewing themselves through cell division, sometimes after long periods of inactivity. Second, under certain physiologic or experimental conditions, they can be induced to differentiate into tissue- or organ-specific cells with special functions. As such, they have the potential to repair and regenerate damaged tissue. The primary mechanism of action is considered to be an immunomodulatory function through a paracrine effect on the surrounding tissue and immune cells, rather than the differentiation and engraftment of the stem cells themselves.

Bio-distribution studies with intravenously injected labelled cells have shown distribution to the lungs, spleen, liver and various other regions, 24 hours after administration. However, only 7 days after administration, cells could hardly be found. After 14 days, all the cells had been cleared from the system. Furthermore, when administered locally, it is generally accepted that stem cells act through paracrine signalling, while long-term persistence or engraftment of the cells may rarely occur. The administered stem cells are therefore very unlikely to be present in food commodities produced from treated animals,

although the presence of subsequent generations of daughter cells cannot be ruled out. More importantly, the presence of such cells is not considered to represent a consumer safety concern as their biological makeup and characteristics will be comparable to those of other routinely ingested cells derived from food producing animals, as will their fate in the consumer (digestion).

Moreover, CVMP considers that a 'No MRL required' classification is appropriate for the following reasons:

- Any food derived from food-producing animals contains many different types of cells (including different types of stem cells), which can be considered normal constituents of the diet in humans and animals. The biological makeup and characteristics of stem cells used in VMPs in food derived from food-producing animals will be comparable to those of other routinely ingested eukaryotic cells, as will their fate in the consumer (digestion). Consequently, the presence of stem cells used in VMPs in food derived from food-producing animals is not considered to represent consumer safety concern different from any other cells. No systemic pharmacological activity is expected due to the fate of the cells after oral ingestion (digestion).
- A primary toxicological concern regarding systemic exposure to stem cells is tumorigenic potential by proliferation due to their capability to self-renew and differentiate into a variety of cell types. Furthermore, immunological reactions might be expected due to systemic exposure to xenogenic cells. However, as laid out above, the cells will not be systemically available unbroken/ undigested, therefore, no toxicological effects based on the characteristics of the stem cells are expected.

Probiotic components including bacteria and yeasts

Probiotic components including bacteria and yeasts have been considered not to fall within the scope of Regulation (EC) No. 470/2009 since 1997. Like stem cells, they were included in the list of substances considered as not falling within the scope of Regulation (EC) No. 470/2009, with regard to residues of veterinary medicinal products in foodstuffs of animal origin (EMA/CVMP/519714/2009), under the heading of Biologically active constituents. With the creation of the list of Chemical-unlike biological substances considered as not requiring an MRL evaluation as per regulation (EU) No. 2018/782, it was considered appropriate to move probiotic components including bacteria and yeasts to that list. The CVMP remains of the opinion that no further MRL assessment is necessary for probiotic components including bacteria and yeasts.

The FAO and WHO have defined probiotics as "live micro-organisms that, when administered in adequate amounts, confer a health benefit on the host". This means that to be recognised as such, probiotics must not have any harmful effects (e.g. by formation of harmful levels of biogenic amines). A substantial array of microorganisms may be considered to fall within this definition, with different microorganisms exerting their effects through different mechanisms, that are not yet fully understood. The FAO/WHO identifies 6 possible mechanisms of action: (i) modification of microbial populations within the gastrointestinal tract, promoting favourable gut microflora, (ii) increasing digestion and absorption of nutrients, (iii) production of antimicrobial substances that may inhibit growth of pathogenic microorganisms in the gut, (iv) altering bacterial communication (quorum sensing), leading to altered gene expression in pathogenic microorganisms, (v) immunomodulation within the gastrointestinal tract and (vi) preventing colonisation of the intestinal mucosa by pathogens organisms (colonisation resistance).

The presence of probiotic microorganisms or their residues in food produced from animals treated with probiotic veterinary medicinal products cannot be ruled out. In fact, consumers are already directly exposed to probiotic microorganisms, either as a result of their presence in the normal diet or as a result of the ingestion of products marketed directly to consumers. However, any probiotics that remain in food commodities produced from treated animals are not considered to represent a consumer safety concern as their biological makeup and characteristics will be comparable to those of other routinely ingested

probiotics, as will their fate in the consumer. It is considered highly unlikely that probiotic VMPs used to treat food producing animals will result in meaningful increases in consumer exposure to relevant microorganisms and their residues.

Moreover, CVMP considers that a 'No MRL required' classification is appropriate for the following reasons:

- Some probiotics are isolated or grouped characterized strains which are commonly found in products marketed directly to consumers and are as such normal constituents of the human diet.
- Probiotics designed for health benefits to animals might not have beneficial health effects to humans because of differences in host physiologic characteristics and the diet. Nevertheless, food derived from food-producing animals is expected to already contain different probiotic components including probiotic bacteria and yeasts. Consequently, their presence in food derived from food-producing animals is not considered to represent a consumer safety concern.

3. Conclusions

Having considered the existing status of bovine casein hydrolysate (bCNH), probiotic components including bacteria and yeasts, recombinant bovine IL-8 (His-tag), stem cells, and varroa destructor calmodulin gene-specific double-stranded interfering RNA EP15 (naked unmodified dsRNA), and in line with the assessment provided above, the CVMP concludes that these chemical-unlike biological substances do not pose a risk to public health and that, consequently, they can be included in Table 1 of the Annex to Regulation (EU) No. 37/2010 in accordance with the following table:

Pharmacologically active Substance	**Marker residue**	**Animal Species**	**MRL**	**Target Tissues**	**Other Provisions**	**Therapeutic Classification**
Bovine casein hydrolysate (bCNH), produced from sodium caseinate hydrolysed with trypsin, heat treated - chemical-unlike biological substance	NOT APPLICABLE	Bovine	No MRL required	NOT APPLICABLE	For intramammary use only	NO ENTRY
Probiotic components including bacteria and yeasts- chemical-unlike biological substance	NOT APPLICABLE	All food producing species	No MRL required	NOT APPLICABLE	NO ENTRY	NO ENTRY
Recombinant bovine IL-8 (His-tag) -	NOT APPLICABLE	Bovine	No MRL required	NOT APPLICABLE	For intrauterine use only at a	NO ENTRY

Pharmacologically active Substance	Marker residue	Animal Species	MRL	Target Tissues	Other Provisions	Therapeutic Classification
chemical-unlike biological substance					dose of up to 1,000 µg per animal	
Stem cells - chemical-unlike biological substance	NOT APPLICABLE	All food producing species	No MRL required	NOT APPLICABLE	NO ENTRY	NO ENTRY
Varroa destructor calmodulin gene-specific double-stranded interfering RNA EP15 (naked unmodified dsRNA) - chemical-unlike biological substance	NOT APPLICABLE	Bees	No MRL required	NOT APPLICABLE	NO ENTRY	NO ENTRY

4. Background information on the procedure

Submission of the request: 13 September 2024

Steps taken for assessment of the substance

Clock started: 11 October 2024

CVMP scientific advice adopted: 15 January 2025