CODEX ALIMENTARIUS COMMISSION

Food and Agriculture Organization of the United Nations

World Health Organization

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PROPOSALS FOR NEW AND/OR REVISION OF FOOD ADDITIVE PROVISIONS

Comments of IFAC

This Conference Room Document (CRD) is respectfully submitted by the International Food Additives Council (IFAC). IFAC appreciates the Committee's consideration of this CRD, which responds to concerns raised by the Russian Federation to the use of nisin as noted in CX/FA16/48/11.

Introduction:

Nisin is a naturally occurring antimicrobial agent produced by some strains of the lactic acid bacterial (LAB) species *Lactococcus lactis*. Originally reported in 1928, nisin has been used as a preservative in a variety of foods since the 1950s. It was approved by the Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) in 1969.

In its response to CL 2015/12-FA, the Russian Federation has raised a number of concerns with the use of nisin in food, which the following information should address.

1. **Justification #1:** Nisin A does not influence the growth of spoiling microorganisms – *Proteus spp., Pseudomonas aeruginosa*, and a lot of number species of lactic-acid-producing bacterium, yeasts and moulds. Yeast and moulds are not only resistant to nisin, but also could quickly destroy this bacteriocin.

   "*nisin A (non bioengineered nisin) has a relatively narrow specter of antimicrobial activity. Nisin exhibits pore-forming activity and the inhibition of cell's membranes biosynthesis of gram-positive microorganisms - Listeria spp., Staphylococcus spp., Bacillus spp., Clostridium spp.*[1,2] *Simultaneously, gram negative microorganisms which are the most important contaminants of ready-to-eat heat-treated meat products (Salmonella spp., Proteus spp., E.coli and another microorganisms of Enterobacteriaceae family, Campylobacter spp.) and caused most cases of food poisoning and acute enteric infections not sensitive to nisin."*

   References:

   [1] Severina E, Severin A, Tomasz A (1998) Antibacterial efficacy of nisin against multidrug-resistant Gram-positive pathogens// J Antimicrob Chemother 41: 341– 347

   [2] FDA (1988) Food and Drug Administration. Nisin preparation: Affirmation of GRAS status as a direct human food ingredient. 11251 ed.

Response: Nisin A displays strong antimicrobial activity against most strains of many Gram-positive bacteria, including important food spoilage microbes such as *Listeria monocytogenes*, *Staphylococcus aureus*, both vegetatively growing cells and spores of *Clostridium botulinum* and *Bacillus cereus* as well as other *Bacillus* species, and LAB that do not produce nisin. Published studies (see Appendix A) provide clear technological justification for use of nisin in various food applications; applications where these spoilage and pathogenic bacteria can and have been documented as the cause for economic losses and endangered public health.

Consumer demand for less processed, more fresh-like and convenience foods, food manufacturers are faced with a strong need to use milder processing methods while ensuring safe, stable and economically viable food products that retain their organoleptic and nutritional quality. Control of microbial growth that can cause spoilage or render a food unsafe can be achieved through the deliberate application and intelligent combination of hurdles such as temperature (high or low), water activity (a_w), acidity (pH), redox potential (Eh), preservatives/antimicrobials e.g. nisin, nitrite, sorbate) and competitive microorganisms (e.g. lactic acid bacteria).

Nisin A is intended to be used in combination with other hurdles to provide a multi-targeted approach to controlling microbial growth in foods produced in conjunction with good manufacturing practice. Nisin A is specifically useful against some microbes that are particularly problematic in foods due to their resistance to other hurdles/preservation methods e.g., cold-tolerant *Listeria monocytogenes*, salt-tolerant *Staphylococcus aureus*, environmentally resistant *Clostridium botulinum* spores.

Nisin A is not intended to be used as a broad spectrum antimicrobial. Consistent with the hurdle technology approach, alternative or additional hurdles should be considered if the intent is to control spoilage and/or pathogenic microorganisms where efficacy of nisin alone has not been demonstrated or validated.

2. Justification #2: It is a verified fact that nisin and another antibiotics have common mechanisms of influence on microbial agent's genome regulation. Therefore using of nisin can promote resistance and increase the risk of transfer of antibiotic resistance to representatives of the intestinal microflora, as well as speeding up virulence and pathogenic potential of microorganisms which cause food borne illnesses.

***a) Item #1*:** *"according to proposals of CCFA elaborated by eWG, prepared in 2014 and in 2015 years, on amendments to the General Standard on Food Additives (Codex STAN 192-1995) nisin could be used in a number of groups of food products, …. These proposals are based on the conclusion of JECFA about safety of nisin.[3] However, in our opinion, JECFA have taken in consideration (as a base) the safety of nisin as chemical substance, but did not take into account possibility of its negative biological effects.*

The EFSA evaluated nisin and endorsed the ADI of 0.13 mg nisin/kg bw per day without taking in consideration its antimicrobial activity.[4]"

References:

[3] NISIN. First draft prepared by First draft prepared by S. Choudhuri , M. DiNovi1 , P. Sinhaseni and J. Srinivasan /Safety evaluation of certain food additives and contaminants. WHO FOOD ADDITIVES. SERIES:68 4 Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids

[4] Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food on the safety in use of nisin as a food additive in an additional category of liquid eggs and on the safety of nisin produced using a modified production process as a food additive /The EFSA Journal (2006) 314b, 1-8

Response: The JEFCA monograph includes analyses and consideration of a broad range of studies relevant to myriad food safety aspects. The "Biological data" section (pp. 92-108) includes subheadings on "Biochemical aspects" (Absorption, Inactivation/degradation, Development of resistance, and Cytotoxicity), "Toxicological studies" (Acute toxicity, Short-term studies of toxicity, Long-term studies of toxicity and carcinogenicity, Genotoxicity, and Reproductive and developmental toxicity), and "Observations in humans." Many of the subsequent comments from the Russian Federation relate to the bacterial development of nisin resistance, which is the focus of the section on "Development of resistance" (pp. 94-95) in the cited reference. This evidence indicates that JECFA was taking the biological effects of nisin A into account in preparing the cited reference.

In 2005, the EU Commission asked the European Food Safety Authority (EFSA) to issue an opinion on the safety in use of nisin. In addition, EFSA was asked to address the issue of antimicrobial resistance and the use of nisin. The published EFSA opinion (1) adopted on January 26th, 2006, includes an extensive discussion on microbiological considerations relating to the mode of action of nisin, the occurrence of nisin resistance and cross-resistance with therapeutic antibiotics. (pp. 6-8) The AFC Panel (Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Foods) concluded that the development of antibiotic resistance to nisin is not of concern in relation to the use in food.

The reference cited by the Russian Federation is a follow-on EFSA opinion to address the expanded use of nisin in liquid eggs and to assess the equivalency of nisin produced via fermentation of a sugar medium as a replacement for the milk medium. It cross references the January 26th, 2006 AFC Panel opinion ion the use of nisin as a food additive.

b) ***Item #2*****:** *"sub-inhibitory concentrations of nisin induced increased resistance of microorganisms in food (for example Staphylococcus aureus) to nisin. These affects were compared with those of vancomycine. Purified nisin is cytotoxic to a number of eukaryitic cell types in vitro in concentration of 0.85-3.4 mmol/l. From the reported studies, the order of nisin's cytotoxity is sperm's cells>red blood cells>SV40-YC cells> Vero cell lines in concentration of 5-640 ppm.[3] "*

Reference:

[3] NISIN. First draft prepared by First draft prepared by S. Choudhuri , M. DiNovi1 , P. Sinhaseni and J. Srinivasan /Safety evaluation of certain food additives and contaminants. WHO FOOD ADDITIVES. SERIES:68 4 Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids

Response: One issue raised in this paragraph is nisin cytotoxicity for mammalian cells. Studies discussed in the cited reference show *in-vitro* cell culture toxicity only at very high nisin levels; much higher than needed to achieve antimicrobial activity and use as a food preservative, and higher than the rare cases in which nisin can be detected systemically after oral administration. For instance, the 2006 Maher & McClean study (2) indicates in the Abstract that "nisin caused hemolysis at concentrations which were 1000-fold higher than those required for antimicrobial activity," and in the Discussion that "nisin … exhibit[s] low cytotoxicity in gastrointestinal cells". More recently Shin et al (3) reported no effects of nisin on viability or proliferation of human cells during in vitro culture except with prolonged exposure to very high concentrations. This natural resistance to nisin shown by mammalian cells is due to the absence of peptidoglycan and its essential biosynthetic molecule Lipid II, a bacterial membrane component which is the binding partner and target responsible for nisin's antimicrobial effects (1-4).

The second issue raised in this paragraph is the microbial development of resistance to nisin following exposure to sub-inhibitory levels. This is a common microbiological phenomenon for antimicrobial compounds, including nisin as well as other food preservatives and antibiotics. Acquired nisin resistance arising under laboratory conditions has been reported for *Bacillus* species, *Clostridium botulinum*, *Enterococcus* species, *Listeria monocytogenes*, *Staphylococcus aureus*, *Streptococcus* species, and other bacteria (1, 7-16). This laboratory finding is entirely predictable and is not inherently a cause for alarm or "red flag" raising concern about food safety or clinically significant antibiotic resistance in human pathogens, for several reasons.

1. Nisin A is a food preservative. It is not approved for clinical therapeutic use in humans (1).
2. Bacterial nisin resistance has generally not been associated with cross-resistance against antibiotics used clinically for treatment of infectious disease (1, 9, 13, 14).
3. The antimicrobial activity of nisin targets lipid and peptidoglycan molecules that are products of complex, multi-step biosynthetic pathways, which are not amenable to the development of resistance via mutation of individual genes. Laboratory acquisition of nisin resistance is typically rare, complex, pleiotropic, and/or unstable (maintained only in the presence of continuous nisin selective pressure). In cases in which the bacterial mechanism(s) for resistance have been examined, it/they have frequently involved changes in the membrane lipid composition or architecture or signal transduction pathways that are only partially understood (1, 5, 7, 8, 11-17).
4. Transmission has not been reported for acquired nisin resistance developed in the laboratory by step-wise exposure to increasing nisin concentrations. The responsible complex membrane or signal transduction alterations may be polygenic or based on epigenetic regulation, features that are not readily transferred between bacteria.
5. Despite widespread use of nisin as a food preservative for more than fifty years, reports of acquisition of resistance in susceptible bacteria have generally been restricted to laboratory studies of pure bacterial cultures under artificial selection conditions rather than "in the field" i.e. in bacteria contaminating foods or colonizing animals as normal microbiota.

c) ***Item #3:*** *"As a result of high biological activities, it has been showed that lantibiotic nisin can potentially be employed as novel antimicrobials preparation to combat medically significant bacteria and their multi-drug resistant forms.[5]"*

Reference:

[5] Perez R.H., Zendo T., Sonomoto K. Novel bacteriocins from lactic acid bacteria (LAB): various structures and applications //Perez et al. Microbial Cell Factories 2014, 13(Suppl 1):S3

Response: The cited reference is a review article discussing fast tracking discovery of novel bacteriocins, the amenability of bacterocins to bioengineering (via genetic modification of the producing microbes) and the opportunities for expanded use of bacteriocins in biopreservation of foods and of bioengineered bacteriocins with enhanced bioactivity, and higher stability in clinical applications, especially against infections caused by antibiotic-resistant pathogens.

Of note is that the authors are supportive of the use of bacteriocins in food applications. Perez et al observed the under-utilization of bacteriocins by the food industry despite a vast array of bacteriocins being discovered in the past two decades and their potential as a solution to the problems of food spoilage and food-borne infections, either alone, or in combination with other methods of preservation. They offered the suggestion that is the combined lack of awareness of what bacteriocins can achieve in food systems, and the lack of enthusiasm to move away from existing food preservation techniques.

Also noteworthy is that Nisin A is not bioengineered, has not been genetically tailored to either increase its activity or target microorganism specificity and is not used clinically in humans.

d) Item #4: *"The higher effectiveness of nisin was showed in case of using as antibacterial substance for treatment in purpose of Clostridium difficily and Listeria monocytogenes growth control.[6,7]"*

References:

[6] Gabrielsen C., Brede D.A., Nes I. F., Diep D. B. Circular Bacteriocins: Biosynthesis and Mode of Action// Applied and Environmental Microbiology, Nov. 2014, Vol. 80, N 22 p. 6854–6862

[7] Campion A. et al. In vivo activity of Nisin A and Nisin V against Listeria monocytogenes in mice //BMC Microbiology 2013, P.13:23

Response: The Gabrielsen at al reference discusses circular bacteriocins; nisin is only mentioned peripherally for comparison and/or as an example in relation to its efficiacy when used in combination with EDTA, its concentration dependent activity and the potential of bioengineering in creating tailor-made peptides for specific applications.

The Campion el al reference discusses the antimicrobial efficacy of nisin A (the best-studied form and the one commonly used as a food preservative) and nisin V (a bioengineered form with a single amino acid change), against *Listeria monocytogenes* both in vitro and in vivo in a mouse model of systemic infection. Although the data shows that both nisin A & V significantly decreased *Listeria* numbers after administration, the experimental conditions used for this publication do not support the therapeutic use of nisin in human clinical medicine. In the study, nisin was administered to mice via the intraperitoneal route which is not a practical route for therapeutic use in humans; the oral or intravenous delivery routes typically used for treatment of human infections. Additionally, intraperitoneal administration in mice avoids pH, solubility, and digestive enzymatic degradation issues typical to oral or intravenous delivery routes. Moreover, intraperitoneal nisin administration occurred only 30 minutes after intraperitoneal inoculation with the infecting *Listeria* bacteria, i.e. the nisin and bacteria were essentially mixed together in the mouse peritoneal cavity.

Nisin A is poorly soluble at human physiological pH and is typically solubilized under acidic conditions; these features are not conducive to intravenous delivery. Nisin A is proteolytically cleaved and inactivated by the pancreatic enzyme-chymotrypsin in the human digestive tract, making oral delivery impractical (1, 5, 18, 19). These attributes partly account for nisin's lack of therapeutic use in humans.

Both references do not experimentally address *Clostridium difficile*. However, in the Campion el al reference a Background section mention of the cited reference: "Currently, a number of lantibiotics are under investigation for clinical use. NVB302, a semi-synthetic derivative of actagardine, is in stage I clinical trials with a view to treat infections caused by the hospital-acquired bacteria *Clostridium difficile*". The direct relevance of this information is not clear for considering the clinical use or impact of the different compound nisin. Perhaps a different reference was intended.

e) Item #5: *"Successful investigations showed higher activity of nisin S, nisin T and nisin V (novel bioengineered derivatives) against M. tuberculosis (H37Ra), M. kansasii (CIT11/06), M. avium subsp. hominissuis (CIT05/03) and M. avium subsp.paratuberculosis (MAP) (ATCC 19698).[8]"*

Reference:

[8] James Carrol et. al. Gene encoded antimicrobial peptides, a template for the design of novel anti-mycobacterial drugs //Bioengineered Bugs, November/December 2010, Vol.1:6, P.408-412

Response: The cited reference demonstrates antimicrobial activity of several bioengineered nisin variants with altered primary amino acid sequence and structure against several pathogenic mycobacterial species. Antibiotic therapy for human infections has challenges, including increasing microbial resistance against existing antibiotics. Consequently, there is considerable interest in identifying new antibiotics, either completely novel compounds or via modification of known antimicrobial compounds. Such attention has included bacteriocins and lantibiotics, classes of compounds of which nisin is a member. Given the antimicrobial efficacy of nisin A, its long history of success as a food preservative, and its designation as "generally regarded as safe" (1, 5, 8, 17), it is unsurprising that there is interest in bioengineering or altering the structure of nisin A in order to achieve compounds with greater antimicrobial activity that also might overcome the obstacles preventing nisin A itself from being therapeutically useful to treat human infectious diseases (discussed above) (17, 20-22). However, these bioengineered variants are different compounds than nisin A. Nisin A is the compound typically used as a food preservative, and is not approved for human clinical applications, due to obstacles which will always hold true for the compound nisin A.

f) Item #6: *"As a result of higher antibacterial activity, bacteriocins are candidates for using in therapy of infection diseases caused by microorganisms with multi-resistance into antibiotics.[9]"*

Reference:

[9] Kruszewska D, et al. Mersacidin eradicates methicillin-resistant Staphylococcus aureus (MRSA) in a mouse rhinitis model // J Antimicrob Chemother, 2004, Vol. 54, P. 648–653

Response: Kruzewska et al's study investigated the antimicrobial activity of mersacidin, a lantibiotic produced by *Bacillus* species, against methicillin-resistant *Staphylococcus aureus* (MRSA), both in vitro and in a mouse intranasal carriage model. A typical pre-clinical exploratory study (i.e. one which demonstrating a starting point for further examination and providing leads that may result in eventual identification of therapeutically useful compounds), it's relevance to assessing the safety or potential clinical impact of using nisin as a food preservative is significantly limited.

First and most importantly, although nisin and mersacidin are classed lantibiotic bacteriocins, mersacidin is a different compound from nisin. In discussing the efficacy of mersacidin as a potentially effective drug to eradicate a nasal human MRSA strain adapted to mice, Kruszewska et al noted "In contrast, another lantibiotic, nisin, has not been successful so far in a similar rodent model…"

Second, like nisin, mersacidin is not currently approved for clinical therapeutic use in humans.

g) Item #7: *"Moreover the higher specificity of some bacteriocin (especially obtained by bioengineering's methods) into multiple-antibiotic resistant species of microorganisms have made them especially attractive as next-generation antibiotics targeting the multiple drug resistant pathogens.[12]"*

"Mechanisms of resistance to nisin of microorganisms Staphylococcus aureus - the most important source of food intoxications,[15] and the Streptococcus bovis which can cause human cancer[16] are under investigation. Resistance to nisin of such serious pathogens as Listeria monocitogenes[17,18] and Clostridium botulinum[19] was noted. Mechanisms of Bacillus subtilis resistance to nisin is investigated. These defensive mechanisms are also effective against other lantibiotics such as mersacidin, gallidermin, and subtilin and comprise an important subset of the intrinsic antibiotic resistome of B. subtilis.[20]"

References:

[12, 15] Miki Kawada-Matsuo et al. Three Distinct Two-Component Systems Are Involved in Resistance to the Class I Bacteriocins, Nukacin ISK-1 and Nisin A, in Staphylococcus aureus //PLOS ONE, www.plosone.org, 2013, Vol. 8 , Is. 7

[16] Hilario C. Mantovani and James B. Russell Nisin Resistance of Streptococcus bovis //Applied and Environmental Microbiology, Feb. 2001, p. 808–813

[17] Teresa M. Bergholz, et al. Nisin Resistance of Listeria monocytogenes Is Increased by Exposure to Salt Stress and Is Mediated via LiaR //Applied and Environmental Microbiology, 2013, Vol. 79 N 18 p. 5682–5688

[18] Barry Collins et al. Assessing the Contributions of the LiaS Histidine Kinase to the Innate Resistance of Listeria monocytogenes to Nisin, Cephalosporins, and Disinfectants //Applied and Environmental Microbiology, 2012, Vol. 78, N 8, p. 2923–2929

[19] Alejandro S. Mazzota et.al., Nisin Resistance in Clostridium botulinum Spores and Vegetative Cells //Applied and Environmental Microbiology, Feb. 1999, p. 659–664

[20] Anthony W. Kingston, Xiaojie Liao, and John D. Helmann Contributions of the σ σ W , σ σ M , and σ σ X Regulons to the Lantibiotic Resistome of Bacillus subtilis //Mol Microbiol., 2013 November, Vol. 90(3), P. 502–518

Response: The 6 cited references provide examples of nisin resistance in a variety of bacteria, all but one of which are associated with disease by either infection or intoxication in humans. Some of the cited references include some degree of characterization of mechanism of nisin resistance on phenotypic or genetic levels.

While report of nisin resistance is quite common in the literature, nisin has remained a very effective food preservative, even against the bacteria that are included in these cited references even after more than five decades of use. Several factors may contribute to this occurrence. First, many of the publications generally report the development of nisin resistance under carefully controlled laboratory conditions rather than in nature or more specifically on foods preserved with nisin. Second, nisin is frequently used as part of hurdle technology including a variety of antimicrobial preservative conditions, thereby reducing the likelihood of development and maintenance of resistance to any one of the components (1, 8, 11, 23). Third, as emphasized by the cited references that address mechanism, nisin resistance is variable among different microbes and typically very complex, pleiotropic, and/or unstable, frequently involving changes in the membrane lipid composition or architecture or signal transduction pathways that are only partially understood; membrane alterations have been speculated to restrict nisin access or pore formation, while signal transduction pathways may have varied effects on bacterial stress responses (1, 5, 7, 8, 11-17). Fourth, bacterial transmission has not been reported for acquired nisin resistance developed in the laboratory by step-wise exposure to increasing nisin concentrations. The responsible complex membrane or signal transduction alterations may be polygenic or based on epigenetic regulation, features that are not readily transferred between bacteria.

h) Item #9: *"It is showed that nisin could be inhibitor of lactobacterium growth which is the most important part of normal gut microbiota. For example, nisin could inhibit a growth of Lactobacillus gasseri in concentration of 25 ng/ml.*[21] *As a result this process can inhibited the non-specific immunity status of population. Antimicrobial peptides play a significant role in building an innate immunity.* [17,22] *The possibility of nisin influence on the activity of specific bacterial enzymes (α- and β-glucosidases, α- galactosidases and β-glucuronidase) in crop, ileum and caeca was taken for the justification of its use in case of chicken breed. The nisin diets supplemented with increasing levels (100, 300, 900 and 2700 IU nisin/g, respectively). At the 35th day counts of Bacteroides and Enterobacteriacae in ileum were significantly (P<0.001) decreased by nisin and salinomycin. Like salinomycin, nisin supplementation improved broiler growth performance in a dose-dependent manner; compared to the nisin group, the body weight gain of the nisin IU=900 and nisin IU=2700 groups was improved by 4.7% and 8.7%, respectively."*[2]

References:

[17] Teresa M. Bergholz, et al. Nisin Resistance of Listeria monocytogenes Is Increased by Exposure to Salt Stress and Is Mediated via LiaR //Applied and Environmental Microbiology, 2013, Vol. 79 N 18 p. 5682–5688

[21] Revilla-Guarinos A., Characterization of a Regulatory Network of Peptide Antibiotic Detoxification Modules in Lactobacillus casei BL23 //Applied and Environmental Microbiology, 2013, Vol.79, N 10, p. 3160–3170

[22] Koczulla, A. R., and R. Bals.. Antimicrobial peptides: current status and therapeutic potential. //Drugs, 2003, Vol. 63, P.389–406

[23] Damian Józefiak et al. Dietary Nisin Modulates the Gastrointestinal Microbial Ecology and Enhances Growth Performance of the Broiler Chickens//PLOS ONE, www.plosone.org, December 2013, Vol. 8, Is.12

Response: Cited reference 21 (Revilla-Guarinos A.) may have been misplaced as it provides another example of complex nisin resistance associated with membrane alterations and signal transduction pathways. It does not describe susceptibility but rather resistance of *Lactobacillus casei*, and it does not include any mention of *Lactobacillus gasseri*.

The larger issue implied by the comment relates to the potential effect of nisin on the natural gut microbiota. Bacteriocins are thought to be widely expressed in the gastrointestinal tract by resident or transient microbiota, and this may contribute to demonstrated or speculated probiotic benefits of LAB including *L. lactis* in the gut and also periurethral area and vagina of animals.

However, when used as a biopreservative in food applications, nisin A ingested via the oral route does not reach the human large intestine where the major human gut microbiota resides. Nisin A is proteolytically cleaved and inactivated by the pancreatic enzyme-chymotrypsin, which is delivered to the upper small intestine (duodenum) via the pancreatic duct (19). Bernborn et al. studied nisin A and its effects in germ-free rats with intestinal microbiota constituted from human fecal microbiota. They reported that nisin A delivered orally to these rats did not alter constituents of the intestinal microbiota and could not be detected in feces in an intact form with antimicrobial activity in a bioassay, though inactive proteolytic degradation fragments could be detected by ELISA (18). Thus, while nisin A has clear utility as a food preservative due to its antimicrobial activity, nisin A ingested in food is cleaved and inactivated by proteases and does not affect the normal gut microbiota (1, 5).

The Józefiak et al study (cited reference 23) was specifically undertaken to explore the efficacy of dietary nisin as a replacement growth supplement for economic benefit. The study demonstrated that nisin exerted a modulating effect on the microbial ecology of broiler chicken gastrointestinal tract (GIT) to improve bird body weight gain. However, the investigators also acknowledged these findings were in sharp contrast to other studies on human microflora-associated rats where dietary nisin had no effect on the gastrointestinal microbial ecology. They suggested that differences in the observations could be linked with dissimilarities in the digestion processes in mammals and birds; particularly digesta passage time (10-13hrs for rats vs 3-5hrs for broiler chickens) and, hence, nisin exposure to different proteolytic enzymes. Furthermore, experimental differences - the duration of the experiments and feed levels could have had an impact as well; the rats were given in two single dosages for two days whereas in Józefiak et al study, nisin was fed at high concentrations to the birds throughout the experimental period of 35 days. Of significance is that the latter feeding regime and purpose of use is very different from the use of nisin in human food where it will be used at comparatively low levels for controlling fool spoilage and/or food pathogen outgrowth.

The point of the innate immunity comment is unclear; cited reference 22 is a review article on various features of antimicrobial peptides (AMPs) including their role in mammalian innate immunity. Production of AMPs is a feature broadly shared across a vast phylogenic spectrum, including bacteria, animals, and plants. Examples of AMPs produced by bacteria (bacteriocins) include lantibiotics such as nisin. Other AMPs (e.g. defensins and cathelicidins) are products of mammalian epithelial, inflammatory, immune and other cell types and are generally recognized as participants in mammalian innate immunity.

Lactic acid bacteria are common to the complex microbiota residing in the mammalian gut. Some of these may produce bacteriocins including nisin that have antimicrobial activity. The normal microbiota including expressed AMPs can also be considered a host defense and part of innate immunity by virtue of resisting or reversing the establishment of microbial pathogens in the human gut or other body environments. These phenomena are different, however, than use of nisin as a food preservative and oral ingestion of small amounts of nisin on foods, which is proteolytically inactivated before reaching the large intestine, site of the largest and most complex human gut microbiota (1, 5, 18, 19).

A separate but more important to the ensuing discussion, the Koczulla and Bals (cited reference 22) review includes the following statements: “The development of microorganisms that are resistant to AMPs is a rare event.[102],” “The reason why resistance to AMP seems to be relatively rare remains speculative. One reason might be that AMPs target structures or processes which are conserved features of the microorganisms and are important for survival and rapid growth.” and “The broad spectrum of activity and the low incidence of bacterial resistance are attractive features of AMPs.” All of these are supportive of the general theme of this response document, which is that development of resistance against nisin is rare and not likely an area of concern based on available evidence.

3. **Justification #3:** Risk assessments and specifications of different types of nisin which were obtained by using biotechnological methods should be provided.

   a) Item #1: ***“****Nisin is also used in the veterinary industry (for example as an anti-mastitis product in the form of Wipe Out®, and an intramammary infusion) and has potential as a clinical antimicrobial.[26]”*

   “However according to established principles medicaments used in clinic cannot be used in food industry.”

   Reference:

   [26] Field D, Begley M., O’Connor P. M. et al. Bioengineered Nisin A Derivatives with Enhanced Activity against Both Gram Positive and Gram Negative Pathogens// PLOS ONE, Oct. 2012, Vol. 7, Is. 10, e46884.

Response: The nisin-based pre-moistened topical wipes (Wipe Out® Dairy Wipes) was first introduced to the US market in 1999 as an antimicrobial wipe to clean and sanitize the teat area before and after milking to manage and reduce the incidence of mastitis in cows. The intramammary infusion cited is a developmental product; nisin-based intramammary treatment of subclinical mastitis in lactating dairy cows; USA FDA approval is still pending. In contrast, nisin A was internationally recognized as safe for use as a food preservative decades earlier.

Because nisin is well characterized, known to be effective against most Gram-positive and has long history of safe use in foods, it is frequently selected as a representative bacteriocin in clinical investigations seeking alternatives to therapeutic antibiotics. In recent years, probiotic bacteria (e.g. lactic acid bacteria) have been proposed as a valid alternative to antibiotic therapies and are also useful for the prevention of infectious syndromes. Lactic acid bacteria exert their effect as a consequence of one or more mechanisms including the production of antagonistic metabolites such as acids, hydrogen peroxide and bacteriocins. (29) As such, use of nisin in this manner could be viewed as an extension or am amplification of its role in nature as a bacteriocin produced by LAB such as *Lactococcus lactis*.

b) Item #2: *"Nisin itself has been subjected to bioengineering for almost twenty years.[28] However only in recent years researchers better understanding of lantibiotic biology and the application of bioengineering strategies on a larger-scale, have achieved notable successes with regard to enhancing the antimicrobial activity of lantibiotics against pathogenic bacteria. Both mersacidin and nukacin have been the subject of comprehensive site-saturation mutagenesis approaches which have resulted in the generation of several novel derivatives with enhanced activity compared to the parent peptide against a range of bacterial targets. It is important to note that this improved activity was strain variable, providing further evidence that nisin derivatives can be generated with distinct target specificities. Thus it should be noted that bacteriocin's activity of nisin obtained from GMO microorganisms is much higher than bacteriocin's activity of nisin A obtained from non GMO microorganisms which was not taken into consideration by JECFA.[3] In this way it is necessary to establish safety records and ML for each type of nisin obtained by using biotechnological methods."*

Reference:

[28] Field D, Begley M., O'Connor P. M. et al. Bioengineered Nisin A Derivatives with Enhanced Activity against Both Gram Positive and Gram Negative Pathogens// PLOS ONE, Oct. 2012, Vol. 7, Is. 10, e46884.

Response: The substance under discussion is nisin A. Nisin A is not bioengineered. The producer culture has not been genetically bioengineered. As such the JECFA review is consistent with the substance under discussion.

4. Justification #4: Based on the data on the possible negative impact nisin on the human health and the fact of bioengineered nisin use it is necessary to conduct the nisin risk revision**.**

"According to it, we suppose it is not reasonable to expend nisin (INS 234) using into new food categories before its risk estimation and risk assessment is implemented. Expanding of nisin using can lead to the development of multi-resistant species of pathogenic and potentially pathogenic microorganisms which are food contaminants, food poisonings and can cause inflammatory disease."

Response: Review of the published literature and analyses of the Russian Federation comments on nisin including cited references lead to the following conclusions:

1. Use of nisin as a food preservative is not likely to have any clinical impact on bacterial pathogens causing human infections.
2. There does not appear to be significant issues with natural development or transmission of antimicrobial resistance arising from nisin use.
3. Available evidence does not support any effect of ingested nisin on the normal human gut microbiota.

Conclusion:

As explained in detail here, the available scientific evidence does not support claims that food uses of nisin lead to antimicrobial resistance. IFAC believes that the information provided here should address the concerns that have been raised by the Russian Federation with the use of nisin in food.

References:

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