Review Article

Probiotics under Selective Pressure: Novel Insights and Biosafety Challenge

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ABSTRACT

The advent of novel high-resolution physicochemical techniques and the integration of omics technologies into biomedical research have opened avenues for investigating the mechanisms underlying bacterial survival in vitro and in vivo, subjected to the influence of biotic and abiotic stressors. This encompasses axenic cultures, microbial communities, and holobionts. The development of innovative methodological platforms has facilitated the acquisition of unique data relevant to both fundamental and applied scientific fields. The experimental results indicated a remarkably high level of genomic plasticity in microorganisms and the potential for the evolution of bacterial virulence under selective pressure. These findings have significantly impacted our understanding of the arsenal of self-defense tools in bacteria and the prioritization of research in this field. The increasing quantity of factual material now necessitates a shift in focus from pathogens to the broader category of commensal bacteria, which are used as probiotics in various fields, including medicine, agriculture, and the food industry. The possibility of large-scale genomic reorganization and progressive evolution of virulence in these bacteria under stressful conditions, as well as their modulation of host cell signaling systems and suppression of innate immunity, negative regulation of key cell cycle controllers, disruption of the structure of the intestinal microbiota and intestinal homeostasis, highlight the obvious insufficiency of our knowledge about the "logic of life" of symbionts and the mechanisms of their interaction with eukaryotic cells. This may compromise the ideas of several practical applications. This underscores the importance of comprehensive studies of commensals, their potential for plasticity in different environmental conditions, and the ways in which they communicate and interact with regulatory networks of higher organisms. It also highlights the need to develop a standardization for assessing the safety of probiotics. The review addresses these issues.

Keywords: Gut Microbes, Probiotics, Selective Pressure, Antimicrobial Resistance, Virulence Evolution.

1. Context

The successful implementation of numerous microbiome research projects as a result of the emergence of innovative technologies has facilitated new avenues for harmonizing living systems in general and establishing guidelines for human health in particular (1). Intestinal commensals and symbionts have become the primary drivers of normal animal and human physiology. The primary agents responsible for regulating the structure of the intestinal microbiota are probiotics, their inducers (prebiotics), and their derivatives (postbiotics). A substantial number of reports on the benefits of probiotics can be found in the scientific literature (2). There is a dearth of comprehensive academic studies on the pros and cons of probiotics, which are necessary to objectively assess their efficacy and safety (3). The mechanisms of interaction between probiotic bacteria and eukaryotic cells have yet to be investigated. Additionally, there is currently no standardization regarding the safety assessment of probiotics, and the regulatory system for probiotics varies from country to country. Nevertheless, these circumstances have not impeded the extensive, unregulated utilization of probiotics in medicine, agriculture, and biotechnology or the formulation of ambitious plans (4).

2. Evidence Acquisition

In light of the bacterial origin of the respective preparations, the evolutionary history of microorganisms, which has shaped their remarkable adaptability to diverse environmental conditions, and the canonical signaling pathways associated with the recognition of foreign antigens in higher eukaryotes, it would not be quite reasonable to postulate the exceptional beneficial role of symbionts and the absence of a "cost of compromise." In this regard, the data on the "shadow side" of beneficial intestinal bacteria—commensals, symbionts, probiotics—obtained as a result of a number of fundamental studies of the molecular machinery of symbiosis, carried out using modern methodological platforms on model systems, did not come as a complete surprise to the academic community. However, the data proved to be significant for correcting ideas about the "logic of life" of intestinal symbionts and the biosafety of probiotics (5-15). A significant amount of data has been obtained for Lactiplantibacillus plantarum, a bacterium with generally recognized as safe (GRAS) status, whose strains are widely used as probiotics. Additionally, the association of this bacterium with Drosophila melanogaster, a classic model organism, has been studied in relation to various molecular aspects, including immunoreactivity and the effects of drugs relevant to animals and humans. L. plantarum is an intestinal symbiont of D. melanogaster. With regard to other organisms, systematic studies are not yet available. Instead, there is only fragmentary information, which, when considered alongside the results of studies of the L. plantarum-D. melanogaster association, indicates an urgent need for comprehensive, large-scale studies of various probiotics and the development of a single global system for their control. This review is dedicated to an examination of novel concepts regarding intestinal commensals, challenges, and prospects for the practical implementation of probiotic strains from the perspective of their biosafety.

3. Results

3.1. Intestinal Commensals and Probiotics vs. Selective Pressure in Antimicrobials: Genome Reorganization and Virulence Evolution

3.1.1. Studies of the Mechanisms of Antibiotic Resistance Development of Bacteria in the 21st Century: Innovative Technologies and New Ideas

Recent experimental data indicates that the adaptation of bacteria to antimicrobials (AMs) under selective pressure may be accompanied by the evolution of the virulence of microorganisms (16, 17). This discovery unveiled another alarming aspect of the antibiotic resistance (ABR) issue. There is evidence that the selective pressure of AMs can induce the appearance of virulence factors (infectivity, adhesion, invasiveness, aggressiveness, and toxigenicity) in harmless environmental microbes, which are referred to as "tritagonists" (commensals or symbionts). Additionally, the selective pressure of AMs can alter the degree of virulence in pathogenic microorganisms, resulting in an increase (progression), weakening (regression), or even disappearance (18). This has highlighted the necessity to direct attention not only to pathogens but also to commensals. It is evident that commensals with GRAS status, which are employed extensively in pharmacology and the food industry as probiotics, necessitate particular scrutiny (19). These bacteria may serve as significant reservoirs and distributors of determinants for ABR and virulence. The advent of cutting-edge high-resolution analytical techniques and the evolution of postgenomic and omics technologies have ushered in a new era of research opportunities in the study of bacterial survival strategies under stress conditions (7, 17, 20). New participants in the mechanisms of antibiotic resistance (ABR) and virulence in bacteria were identified, and the understanding of the molecular machinery of microbial adaptation to antimicrobial agents (AMs) was expanded. It was thus found that (i) the mechanisms of ABR development are not limited to those described earlier, namely, those that were identified in the pre-genomic era (21, 22); (ii) ABR is not always associated with mutations in target proteins, but is accompanied by multiple changes in the genomic, transcriptomic, and proteomic profiles that determine significant rearrangements in the bacterium's metabolism, which may result in a change in the virulence status of bacteria (22). (iii) Extracellular vesicles (EVs) are membrane nanostructures secreted by bacterial cells that transport a range of compounds, including lipids, proteins, polysaccharides, DNA, RNA, and small regulatory RNAs. They play a crucial role in adaptive resistance to AMs and virulence realization, facilitating cell-to-cell communication and interactions with pro- and eukaryotic cells (23, 24). The

majority of publications on this topic focus on clinically significant bacteria and facultative pathogens. In the study of probiotic bacteria, only preliminary steps have been taken in this direction thus far.

3.1.2. ABR in Intestinal Commensals with GRAS Status: Change of Genomic Profile and Phenotypic Resistance in a Probiotic Strain of *L. plantarum*

The analysis of probiotic bacteria with respect to modules defined as the resistome, mobilome, and virulome is currently a significant aspect of assessing the safety of these bacteria (25). The analysis of the functions of extracellular vesicles of probiotics is an assessment of the beneficial properties of these nanostructures for potential practical applications (26). The first and currently only available study to assess the possibility of modulating the genomic profile for the virulome, mobilome, and resistome, as well as phenotypic antibiotic susceptibility and virulence in vivo in a probiotic strain when adapting to antibiotics, was conducted in our research on the L. plantarum model (6). As a result of the selection process, a strain of L. plantarum 8p-a3-Clr-Amx was obtained from the L. plantarum 8p-a3 strain, which was isolated from the probiotic Lactobacterin (Biomed, Russia). The mutant strain demonstrated increased resistance to both amoxicillin and clarithromycin (antibiotics commonly used to eradicate H. pylori) compared to the parent strain (MIK8p-a3-Clr-Amx – 20 μg/ml and 10 μg/ml, and MIK8p-a3 0.5 μg/ml and 0.05 μg/ml, respectively). The development of resistance to relevant antimicrobials in L. plantarum in vitro has been found to be accompanied by significant changes in both the genomic profile and phenotypic susceptibility to a number of antimicrobials. These changes include point mutations, as well as deletions, insertions, duplications, and DNA sequence intragenomic transfers, which are associated in part with the resistome and mobilome. Conversely, the results of the phenotypic resistance profile of L. plantarum strains (8p-a3 and 8p-a3-Clr-Amx) exhibited notable discrepancies from the data obtained from the genomic analysis in silico. The 19 mutations identified in the genome of L. plantarum 8p-a3-Clr-Amx had not previously been described as the underlying causes of antibiotic resistance. This suggests that the mechanisms of adaptation to antimicrobials in L. plantarum are not restricted to those previously described. Similarly, Cao et al. (22) employed an integrative approach based on comparative genomics, proteomics, and reverse genetics of antibiotic-adapted strains in the laboratory to investigate the mechanisms underlying the development of L. plantarum P-8 resistance to ampicillin. Similarly, Anisimova et al. (7) reached analogous conclusions with respect to a number of other strains in their analysis of antibiotic sensitivity in diverse commercial probiotic preparations.

3.1.3. ABR of Intestinal Commensals with GRAS Status: Evolution of Virulence in a Probiotic Strain of *L. plantarum*

The conclusions that the mechanisms of adaptation to antimicrobials are more complex than was previously thought in the "pre-genomic era" and that they are not always associated with mutations in the genes of target proteins are not new. These conclusions were made on the basis of studies of different species of bacteria (17, 21, 27). However, in the case of probiotics, this fact is of particular relevance for the analysis of their safety, the presence of determinants of ABR (which can potentially be transmitted to other bacteria, including pathogens) and the possibility of virulence. Our studies demonstrated that the adaptation of the probiotic strain of L. plantarum to antimicrobial agents (AMs) is accompanied by genomic reorganization and the evolution of bacterial virulence. Consequently, the oral administration of L. plantarum 8p-a3-Clr-Amx had a detrimental impact on (i) viability, (ii) reproduction, (iii) the structure of the intestinal microbiota, and (iv) the intestine of fruit flies. The antibiotic-resistant strain demonstrated a high level of toxigenicity, including genotoxicity, and induced a high level of single-strand DNA breaks in fly enterocytes and intestinal necrosis (6). A notable finding was that L. plantarum 8p-a3, the original parent strain, also exhibited negative properties, albeit to a lesser extent than L. plantarum 8p-a3-Clr-Amx. However, its effects were more pronounced than those observed in the resident strain of L. plantarum DMC-S1, which was isolated from the intestines of uninfected flies and did not exert any detrimental effects on Drosophila. The RR values indicated a positive association of the bacterium with the host. The virulence of L. plantarum (KP, DF) in adult flies, associated with the negative effect of the bacterium on the intestine of D. melanogaster, was also observed by Fast et al. (28). This suggests that the relationship between the bacterium and Drosophila is not straightforward. The species L. plantarum is known to demonstrate a high level of heterogeneity and adaptability, manifesting its presence across an array of ecological habitats. These include humans, animals, and plants, as well as dairy, meat, and fish, in addition to vegetable or plant fermentations (Figure 1). It remains unclear whether the heterogeneity of L. plantarum strains, the distinctive characteristics of the bacterial status in terms of genomic profile, as well as D. melanogaster lines, the conditions of cultivation of flies, and the structure of their intestinal microbiota, influence the outcome of the cross-talk between micro- and macroorganisms. It is evident that further investigation is required to elucidate the mechanisms underlying the progressive evolution of virulence in the probiotic strain of L. plantarum during its adaptation to antibiotics. Additionally, a comprehensive assessment of the safety of the commercial preparation is imperative.

3.1.4. ABR and Safety Assessment of Intestinal Commensals with GRAS Status: *in silico* Genomic Analysis and the Problem of Incomplete Gene Annotation

It is postulated that the pool of critical genes that determine the resistome, mobilome, and virulome in L. plantarum encompasses 41 genes (29).

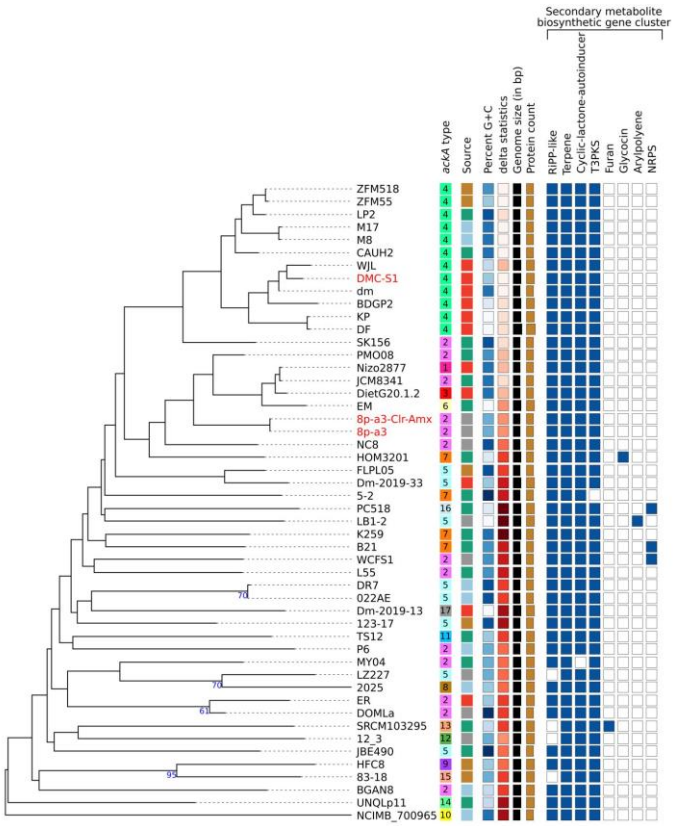

Figure 1. The phylogenomic tree of L. plantarum strains isolated from disparate sources is based on genome-wide data. The numbers adjacent to the branches are GBDP pseudo-load support values exceeding 60% in 100 replications, with an average branch support value of 14.6%. The sources of bacterial excretion are as follows: red – D. melanogaster, brown – human faeces, green – fermented foods, blue – dairy products, gray – other sources. A phylogenomic tree was constructed using the GGDC web server (http://ggdc.dsmz.de/).

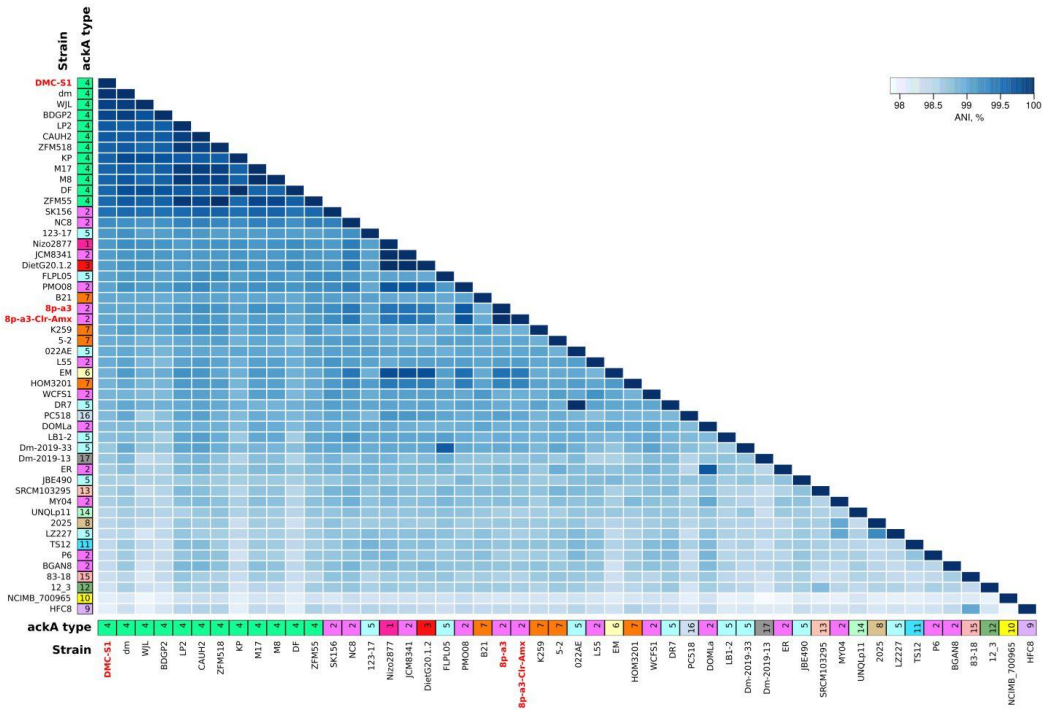

Figure 2. Heatmap of pairwise average nucleotide identity (ANI) among 50 genomes of *L. plantarum* strains (including DMC-S1 and 8p-a3 isolated from *D. melanogaster* and the commercial probiotic "Lactobacterin", respectively).

Of the aforementioned genes, only those encoding integrases (int) are present in the genomes of L. plantarum strains 8p-a3 and 8p-a3-Clr-Amx. Indeed, this does not permit the original strain of L. plantarum 8p-a3 to be regarded as entirely secure. The documented integration of the int gene into the gene for esterase during the bacterium's adaptation to AMs (6) substantiates the potential risks associated with the presence of mobile elements in probiotics. Nevertheless, it seems implausible that the sole determinant of virulence and its evolution during the adaptation of the bacterium to antibiotics is this gene. It is possible that alterations in the primary structure and/or expression of other genes may contribute to the virulence of the commensal (7,10,22,30). Recently, it was demonstrated that the L. plantarum ΔmprF mutant exhibited a deficiency in the synthesis of lysyl-phosphatidylglycerol (Lys-PG) and, in comparison to the wild-type L. plantarum, induced a more pronounced intestinal immune response in flies due to the increased release of immunostimulatory peptidoglycan fragments. This indicates that MprF plays an important role in promoting the reduced level of effectors that impair host cells (10). A mutation in mprF, which caused the transformation of this gene into a pseudogene, was identified in L. plantarum 8p-a3 (6). It is yet to be determined whether the evolution of virulence in the strain is related to the phenomenon. Nevertheless, despite the utility of mutagenesis variants in verifying the involvement of relevant proteins in the virulence of L. plantarum and its progressive evolution of virulence during the adaptation of the bacterium to antibiotics, a considerable number of genes, including strain-specific ones that have not yet been annotated, will stay out of sight (Figure 3). The lack of comprehensive annotation of genes in bacterial genome sequences, particularly in the context of probiotic strains, is a significant concern. In the absence of knowledge regarding the nature and markers of bacterium virulence, the safety of the microbe in question becomes a significant challenge to control.

3.1.5. Adaptation of Probiotics to Environmental Conditions: Multiple ABR, Horizontal Gene Transfer in Bacteria, and Negative Host Reactions

The data on the ability of probiotics to acquire and spread (*via* horizontal transfer) the determinants of resistance, as well as other genetic elements important for survival in aggressive environments and microbial communities, have been described in *Lactobacillus paracasei, L. rhamnosus, L. reuteri, L. gasseri, L. plantarum,* as well as some other bacteria with GRAS status. These data have been summarized in reviews by Lerner et al. (4). Alarming reports of the presence of phenotypic multiple resistance and discrepancies between the resistance phenotype and genotype in probiotics of *L. helveticus, L. plantarum, L. paracasei, and L. fermentum* isolated from different commercial preparations, (as well as a discrepancy between the manufacturers' claims and the established species composition, the indicated number of viable bacteria) were recently presented by Anisimova et al. (7). A substantial body of evidence suggests that the use of different probiotics may have adverse effects. These include increased bacterial adhesion, induction of hemolysis, mucolysis, DNA degradation, and proteolysis in target cells, as well as modulation of the microbiota and activation of mobile genetic elements. The reviews by Lerner et al. (4) and Singh et al. (31) provide a comprehensive summary of the adverse effects associated with the hyperactivation of the immune system and bacterial overgrowth in the gut, including the development of bacteremia, fungemia, sepsis, endocarditis, meningitis, pneumonia, peritonitis, intestinal ischemia, and several other conditions. Nevertheless, there is a dearth of systematic and comprehensive studies examining the safety of bacterial agents, including the characterization of antibiotic resistance (ABR) status, that employ a range of modern methodological platforms and model systems, both in vivo and in vitro, as well as at the cellular level. These studies are absent for any probiotic. With regard to the knowledge base concerning the range of strategies that probiotics can employ to adapt to AMs, the nature and evolution of virulence, reliable molecular markers of ABR status and virulence, there are notable gaps in our understanding. In light of these considerations, the current regulatory vacuum in the probiotics industry appears to be a logical shortcoming. The principal conclusion to be drawn from the data presented in this section is that our understanding of the survival potential of bacteria with GRAS status under different environmental conditions is woefully inadequate. The plasticity of these bacteria, coupled with their capacity for adaptation to antimicrobials, is a phenomenon that defies expectations. However, there is a paucity of systematic studies examining the adaptation of diverse probiotics to varying environmental conditions. The study on the L. plantarum model and the association of this bacterium with D. melanogaster represents a preliminary step in this direction. With regard to other bacteria, however, there is only fragmentary information, and the alarming facts that emerge from this situation highlight the urgent need to (i) conduct large-scale, standardized studies on the mechanisms for ABR and virulence in different probiotics, and (ii) develop an assessment of their safety.

3.2. Commensals vs. Host Gut: Immunomodulation and Proliferation

3.2.1. Conservative Mechanisms of Immunoreactivity in Higher Eukaryotes to Intestinal Commensals Cause Negative Effects for Both Bacteria and the Host

Drosophila employs two distinct mechanisms to defend against pathogens: the cellular and humoral arms of the immune system. Hemocytes engage in cellular immune defense, while reactive oxidative species (ROS) and antimicrobial peptides (AMPs) are involved in the humoral immune response. The control of microbes in the intestine of fruit flies is primarily achieved through the action of ROS and AMP, which are produced as a consequence of the activation of NOX/DUOX complexes and a number of signaling cascades, including those associated with IMD, Toll, ILS, TOR, JNK, and JAK/STAT (Figure 4).

COG Category	Strains of *L. plantarum* DMC-S1	Dmel[1]	DR7	EM	8p-a3	8p-a3-Clr-Amx
Cell cycle control, cell division, chromosome partitioning (D)	1	3	4	21	1	1
Cell wall/membrane/envelope biogenesis (M)	1	9	15	45	17	17
Multidrug efflux system permease protein	0	0	0	1	0	0
Lactococcin A secretion protein LcnD	1	0	0	0	0	0
Cell Motility (N)	0	1	0	6	2	2
Posttranslational modification, protein turnover, chaperones (O)	0	0	0	1	1	1
Signal transduction mechanisms (T)	0	2	0	1	1	1
Endoribonuclease toxin MazF	0	0	0	1	0	0
Intracellular trafficking, secretion, and vesicular transport (U)	1	5	1	13	6	6
Defense mechanisms (V)	4	1	4	5	8	8
Multidrug resistance ABC transporter ATP-binding and permease protein	0	1	0	0	0	0
Putative ABC transporter ATP-binding protein	0	1	0	0	0	0
ABC transporter ATP-binding protein YtrB	1	0	0	0	0	0
Lactococcin-G-processing and transport ATP-binding protein LagD	1	0	0	0	0	0
Chloramphenicol acetyltransferase	0	0	1	0	0	0
Daunorubicin/doxorubicin resistance ATP-binding protein DrrA	0	0	0	1	0	0
Translation, ribosomal structure and biogenesis (J)	1	3	1	0	1	1
Transcription (K)	3	22	16	15	12	12
Replication, recombination and repair (L)	8	33	25	130	24	24
Energy production and conversion (C)	0	0	11	11	1	1
Amino acid transport and metabolism (E)	1	10	10	22	5	5
Nucleotide transport and metabolism (F)	0	3	1	1	0	0
Carbohydrate transport and metabolism (G)	3	9	20	38	11	13
Coenzyme transport and metabolism (H)	0	4	5	3	1	1
Lipid transport and metabolism (I)	0	2	4	5	2	2
Inorganic ion transport and metabolism (P)	1	13	17	13	7	7
Putative multidrug resistance protein MdtD	1	0	1	0	1	1
Multidrug resistance protein 3	0	0	1	0	0	0
Enterobactin exporter EntS	0	0	1	0	0	0
Tetracycline resistance protein, class B	0	1	0	0	0	0
Secondary metabolites biosynthesis, transport and catabolism (Q)	0	1	2	2	1	1
Function unknown (S)	37	166	147	271	140	140
Intracellular iron chaperone frataxin	0	0	1	0	0	0

Figure 3. The distribution of proteins encoded by strain-specific genes in L. plantarum isolated from D. melanogaster, commercial probiotic "Lactobacterin," and other sources is presented according to functional categories. The The Dmel[1] strain is a combination of four strains isolated from D. melanogaster (KP, DF, dm, and BDGP2). The source of DMC-S1 is *D. melanogaster*, 8p-a3, 8p-a3-Clr-Amx - human, DR7 - dairy products, EM - fermented products.

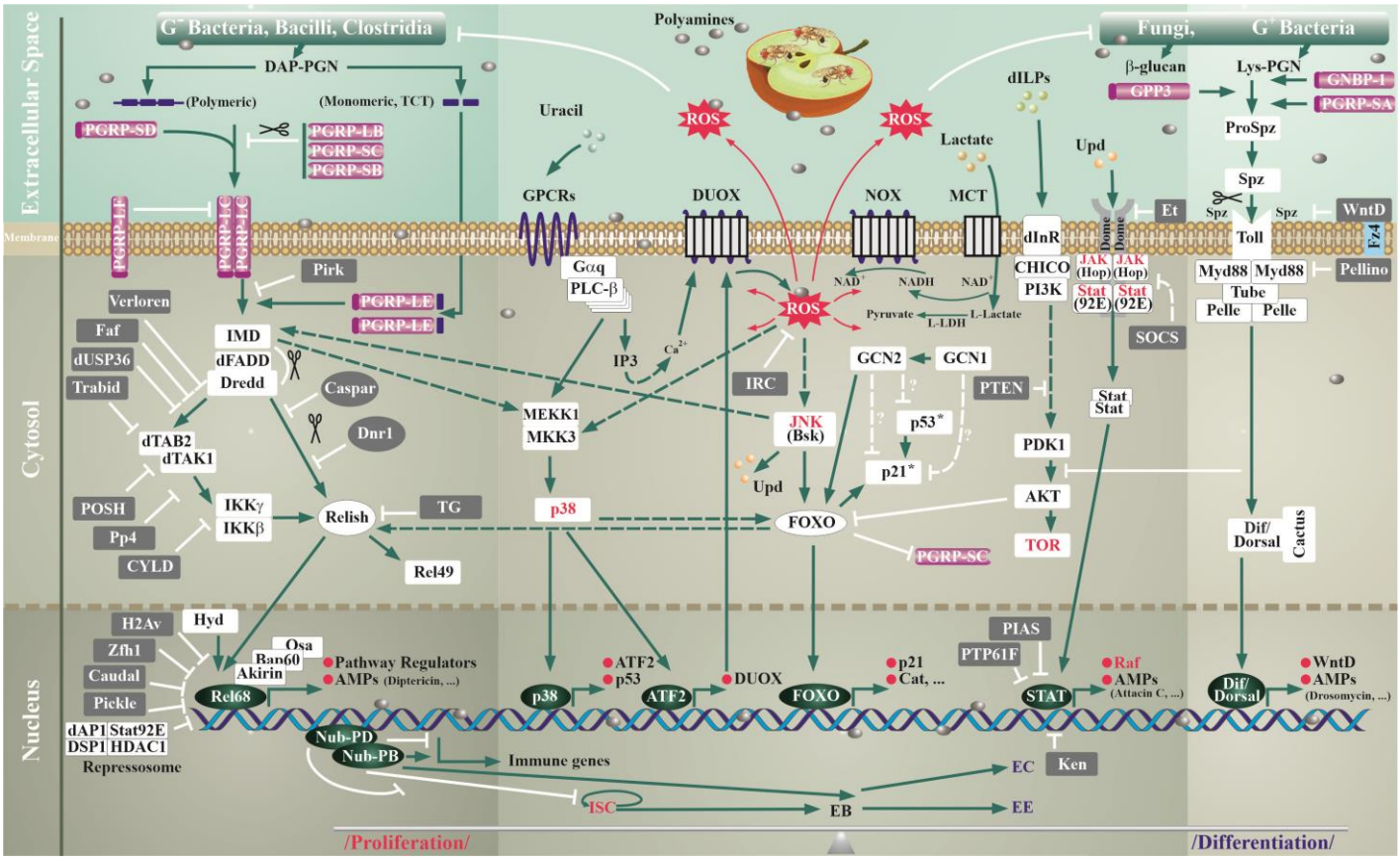

Figure 4. The primary cell signaling pathways that regulate the microbiota and gut homeostasis in Drosophila melanogaster are illustrated in this simplified schematic representation. The IMD and Toll signaling pathways are implicated in the recognition of microorganisms and the determination of effector reactions in Drosophila. The Toll and IMD pathways are activated by the PGN of microbes, specifically the Lys and DAP types, respectively. Additionally, the Toll pathway is activated by the β-1,3-glucan of fungi. Different types of PGN are recognized by different types of transmembrane and/or intracellular PGRPs. It should be noted that effector expression can be induced not only by microbes, but also by other signals, including various biotic and abiotic stressors, metabolic shifts, and age-related changes. It is postulated that the isoforms of the Nubbin transcription factor (Nub-PB and Nub-PD) serve as the primary regulators of immune and intestinal homeostasis. Polyamines are emerging as regulators of intestinal epithelial renewal and barrier function. Polyamines regulate the expression of genes encoding proteins involved in growth through a variety of mechanisms, including the binding of RNA and the action of non-coding RNAs. In addition to endogenous biosynthesis, the gut microbiota, including strains of Lactobacillus, represents a significant source of luminal polyamines. Akirin - NF-B co-factor required for the activation of a subset of Relish-dependent genes, characterized by the presence of the H3K4ac epigenetic mark;Akt - protein kinase B (PKB); AMPs - antimicrobial peptides; Ask1 - apoptotic signal-regulating kinase 1; ATF2 - activating transcription factor 2;BAP - Brahma-associated protein SWI/SNF chromatin-remodeling complex; Bsk - Basket; Cactus – IκB- like protein; Caspar - ubiquitin-related domain bearing protein; Caudal - transcription factor of the homeobox family Caudal;Chico - insulin receptor substrates; CYLD - deubiquitinating enzyme cylindromatosis; NOX- NADPH oxidase; DUOX - dual oxidase; IRC- immune-reactive catalase; Upd3 – unpaired family protein 3, orthologue of Interleukin-6; Upds - unpaired family of cytokine-like proteins; EB-enteroblasts EE – enteroendocrine cells; ESCs - intestinal stem cells; dAP-1 - Drosophila activator protein 1, specific transcription factors of the JNK pathway; Dif - transcription factor Dorsal-related immunity factor; dILPs – drosophila insulin-like peptides;Dnr1 - defense repressor 1, RING-finger containing protein; Dorsal - transcription factor Dorsal; Dredd, Death related ced-3/Nedd2-like caspase (also known as Dcp2); DSP1- Dorsal switch protein 1; dUSP36 - ubiquitin-specific protease 36; Eff - Effete (E2 ubiquitin-conjugating enzyme, an Ubc5 homolog); Et – Eye transformer, negatively regulator JAK-STAT pathway; Fadd - Fas-associated death domain; Faf - deubiquitinase Fat facets (faf); Foxo - transcription factor Forkhead box O; Fz4 – receptor Frizzled4 (class of unconventional GPCRs) inhibits the Toll pathway when it binds the ligand WntD; GCN1 - General control nonderepressible 1kinase; GCN2 - General control nonderepressible 2 kinase; GNBPs – Gramnegative (bacteria) binding proteins GPCRs - G protein-coupled receptors; H2Av – unconventional histone variant; HDAC1-histone deacetylase; Hop - the receptor-associated Janus kinase Hopscotch; Hyd - E3 ubiquitin ligase; IKK *β* - Inhibitor of NF-B Kinase catalytic subunit beta; IKK γ - Inhibitor of NF-B Kinase regulatory subunit gamma; Imd,- Immune deficiency; IP3 - inositol tris-phosphate; JNK - c-Jun N-terminal kinase; JAK/ Janus kinase; MCT - *monocarboxylate transporter;*Mkk3 - MAP kinase kinase 3; Myd88 - adaptor protein Myeloid differentiation primary response gene 88; Nub –PB - Nubbin transcription factor PB-isoform; Nub –PD - Nubbin transcription factor PD-isoform; p38 - MAP kinase superfamily stress-activated serine/threonine protein kinase; Pdk1 - 3-phosphoinositide dependent protein kinase-1; Pelle - the serine/threonine kinase ortholog of IRAK; Pellino - a RING-domain-containing ubiquitin E3 ligase; PGN - peptidoglycan; PGRP-LC, GRP-LE - Peptidoglycan recognition, ligand binding proteins; PGRP-LF - constitutively activated protein that does not bind PGN, but prevents multimerization of PGRP-SC, PGRP-LB, PGRP-SB - secreted molecules, amidase enzymes that cleave the ligand; PIAS – Protein Inhibitor of Activated STAT; Ken – Ken and Barbie; PI3K - phosphatidylinositol 3-kinase consisting of two subunits, catalytic Pi3K92E (Dp110) and regulatory Pi3K21B (Dp60); PIP3 - phosphatidylinositol 3,4,5-trisphosphate; Pirk - Poor Imd response upon knock-in; Pickle - a nuclear IκB factor, also named Charon; PLC-*β phospholipase C-beta;* POSH - Plenty of SH3s (POSH), an E3 ubiquitin ligase, PP4 - protein phosphatase; ProSpz – pro-protein Spätzle, is processed to a functional form by a serine protease; PTEN - phosphatase and tensin homolog; PTP61F – Protein Tyrosine Phosphatase 61F; Raf – serine/threonine-protein kinase(proto- oncogene), involved in the control of cell proliferation and differentiation; Rel - transcription factor Relish; SOCS – Suppressor of cytokine signaling; Spätzle **,** dimeric ligand that responds to the Gram-positive bacterial or fungal infection by binding Toll receptor; is synthesized as a pro-protein and is processed to a functional form by a serine protease; STAT -Signal transducer and activator of transcription; Stat92E - Signal-transducer and activator of transcription protein at 92E; specific transcription factors of the JAK/STAT pathway; Tab2 - Tak1-associated binding protein 2; Tak1 - Transforming growth factor (TGF-)-activating kinase 1; Tg - Transglutaminase; Trabid - deubiquitinase, also known as ZRANB1; Tor - Target of rapamycin kinase; Tube - adaptor protein Tube; Uev1A - Ubiquitin-conjugating enzyme variant 1A (Ubc/E2 variant (Uev) homolog); Verloren - SUMO-specific protease; WntD – The Wnt family ligand WntD provides a buffering system for variations in Toll signaling between embryos. Zfh1 is a zinc finger homeodomain transcription factor. The role of negative regulators is indicated by the use of white lines. The dotted white lines with a question mark are shown for mammals in cellulo and in vivo. Proteins and factors that activate cell proliferation are indicated in red. The asterisks indicate negative regulators of cell proliferation.

The recognition of bacteria in the intestines of Drosophila is primarily associated with the IMD pathway. The effectors (ROS and AMP) exert their influence not only on microorganisms but also on host cells. Damage to intestinal cells stimulates the proliferation of ISCs, and excessive proliferation (in the case of increased immunoreactivity due to host characteristics and/or a high microbial load) leads to dysplasia, disruption of intestinal homeostasis, and a decrease in the lifespan of flies. The administration of antibiotics has been demonstrated to significantly prolong the lifespan of animals by inhibiting the growth of microbes. An overgrowth of commensal bacteria in the gut may be associated with overfeeding and/or aberrant expression patterns of host immunocontrollers. The interconnection of IMD with other signaling systems (Toll, ILS, TOR, JNK, JAK/STAT), genetically or epigenetically mediated features of the expression of immunosensors, adapters, and effectors, as well as positive and negative regulators of signaling cascades, affect the outcome of events. Abnormalities in the expression of immunoregulatory factors result in alterations to the structure of the intestinal microbiota, which in turn lead to a reduction in the lifespan of the flies. This pattern is also observed in major facultative symbionts. For example, the high abundance of L. plantarum in the Drosophila gut has been demonstrated to exert a deleterious effect on intestinal homeostasis and the lifespan of the flies. This phenomenon can be attributed, at least in part, to the intrinsic characteristics of the bacterium in question, specifically its capacity to produce L-lactate, which contributes to the generation of ROS. The potential for a high prevalence of L. plantarum in the Drosophila melanogaster intestine is contingent upon the bacterium's capacity to resist antimicrobial peptides (AMPs), evade the host immune response, and suppress immune sensors. This may be attributed to the bacterium's possession of specific mechanisms. The resistance to cationic antimicrobial peptides (AMPs) and the evasion of the host immune response observed in commensals may be related to the modification of the bacterial cell surface (wall), mediated expression of MprF or Ltd. This has been demonstrated by the model L. plantarum-D. melanogaster interactions (9, 10). PGRP-SC is regarded as a pivotal negative regulator of L. plantarum in the Drosophila intestinal tract. Recently, data have been obtained indicating that L. plantarum possesses a specific mechanism for regulating this immunosensor.

3.2.2. Intestinal Commensals Modulate Host Immunosensors: Mutations in the *ack*A Gene of *L. plantarum* May Favor Host and Bacterial Cell Proliferation

A series of studies were conducted using a combination of classical methods and innovative approaches to create mutant lines of D. melanogaster and L. plantarum, and to analyze the interactions between the two. It has been demonstrated that mutations (with loss of function) can occur in L. plantarum under conditions of selective pressure (when co-cultured with D. melanogaster and/or adaptation to the Drosophila diet) in the ackA gene, resulting in a change in the bacterium's metabolic processes. The interaction between larvae and the corresponding mutant Lactobacillus lineages results in the specific modulation of gene expression, proliferation of the corresponding bacteria in the intestine, and stimulation of growth in fruit flies. A mutation in the ackA gene that confers a beneficial effect on the bacterium is accompanied by an increase in the production of N-acetylated amino acids, including N-acetylglutamine (NAG), in L. plantarum cells. The alteration in the bacterium has been demonstrated to have a stimulatory impact on the growth of Drosophila larvae. In larvae colonized with the corresponding bacteria, two notable effects were observed: (i) increased proliferation of lactobacillus in the intestine and (ii) modulation of the expression of a number of genes involved in various biological processes, including stress response, proteolysis, fertility, and lifespan, as well as recognition and hydrolysis of PGN. Additionally, the expression of the gene for PGRP-SC, a negative regulator of the proliferation of several intestinal commensals, including L. plantarum, was found to be suppressed. The authors demonstrated that the inhibition of the PGRP-SC gene expression in Drosophila can be induced by NAG. The introduction of this metabolite is sufficient to suppress the expression of the PGRP-SC gene and improve larval growth in the presence of L. plantarum strains that do not have a corresponding mutation in the ackA gene. PGRP-SC is a member of the group of amidases that results in the fragmentation of PGN, destruction of the bacterial cell wall, and prevention of the activation of the IMD pathway (Figure 4). DAP-PGN from L. plantarum has been demonstrated to activate the IMD pathway. In light of these considerations, it was anticipated that the proliferation of L. plantarum in the intestines of larvae, occurring concurrently with the suppression of PGRP-SC gene expression, would give rise to alterations in the expression of genes encoding key regulators and effectors of the IMD pathway. These genes are involved in the recognition of PGN (produced by multiply bacteria) by PGRP-LE, PGRP-SD, and PGRP-LC receptors. Nevertheless, no alterations in the expression of markers associated with immune pathway activation (as part of the transcriptional assay) were observed. The molecular basis of this phenomenon remains unclear, as does the reactivity of general stress regulators of immune and intestinal homeostasis in Drosophila, including Nub-PD and Nub-PB. A thorough understanding of the molecular mechanisms underlying this phenomenon is essential for comprehending the potential costs associated with the "beneficence" of a strain carrying a specific version of the ackA gene. In light of the following considerations: (i) the conservatism of mechanisms of microbial immunoreactivity, (ii) the negative effects on the host when there is a high level of abundance of L.plantarum , and (iii) the data on increased mthl6 gene expression in larvae when associated with the corresponding Lactobacillus strain (12),

the analysis of the intestinal state (including the mitotic index of enterocytes and DNA damage) at different stages of the Drosophila life cycle, as well as the lifespan of individuals, appear to be highly relevant for assessing the safety of this bacterium. The fact that mutations can occur in the genome of intestinal commensals under conditions of selective pressure, resulting in changes in bacterial metabolism, indicates the presence of a dangerous property in a bacterium with GRAS status. These mutations can determine changes in the metabolism of bacteria, allowing microbial metabolites to modulate the expression of host genes and suppress immunosensors. This provides opportunities for bacterial expansion. In accordance with the metabolic processes observed in conventional bacterial cells, the ackA deletion has the potential to significantly alter the concentration of acetyl-phosphate (AcP), a signaling metabolite that can transfer phosphate groups to regulatory proteins, thereby modulating the expression of numerous genes or influencing other biological processes. Furthermore, AcP plays a role in the acetylation of enzymes and regulatory proteins, with broad physiological implications (32). In this regard, the physiology and virulence of bacterial strains with an ackA gene mutation that results in a "loss of function" may differ significantly from that of strains with other versions. It remains to be seen whether this is the case with L. plantarum.

3.2.3. *L. plantarum* Strains With a Mutation In the *ack*A Gene That Promotes Host-Bacterium Cell Proliferation Are Widespread

The NCBI database contains 17 variants of the nucleotide sequence of the ackA gene in L. plantarum strains. The gene is highly conserved, with all 17 variants exhibiting a single point of divergence, resulting in a difference of 1-2 mutations (Figure 5). In the studies conducted by the authors (12), a number of strains differing in the ackA sequence were utilized. (1) NIZO2877 is the original strain, which exhibits a moderate Drosophila-growth-promoting ability (ackA sequence variant No. 1); (2) FlyG2.1.8 was obtained through experimental evolution in poor-nutrient conditions in the presence of D. melanogaster derived from NIZO2877. This strain displays an enhanced ability to promote Drosophila growth, with a modified ackA gene that has undergone a deletion of amino acid 345 (alanine), resulting in a loss of function (ackA sequence variant No. 2); (3) DietG20.2. 2 – experimentally evolved on the fly diet from NIZO2877, improved growth promotion, in the ackA gene – replacement of glutamic acid with lysine at position 333, loss of function (ackA sequence variant No. (3) WJL, which is phylogenetically distinct from the preceding strains, differs from NIZO2877 by the substitution of glutamine for arginine at position 58 and the substitution of valine for isoleucine at position 157 (ackA sequence variant 4). (4) ΔackA is NIZO2877 without the ackA gene. The NCBI database contains six variants of the ackA gene sequence in 24 L. plantarum strains isolated from Drosophila. Concurrently, nine strains (including DMC-S1, isolated from the intestinal microbiota of D. melanogaster and exhibiting a positive association with Drosophila) exhibit variant No. 4 of the ackA gene sequence. This variant has also been identified in a number of L. plantarum strains isolated from a variety of sources, including humans and fermented foods (Figure 1). With regard to the "beneficial" sequence (variant No. 2), it was discovered to be exceedingly prevalent, with over half of the strains whose genomes are represented in the NCBI database exhibiting this version of the ackA gene. This variant has been identified in eight strains isolated from Drosophila, as well as in strains from other sources, predominantly dairy products, fermented foods, and the human body. It was an unexpected finding that the cohort of strains with version 2 also included L. plantarum 8p-a3 and 8p-a3-Clr-Amx, for which virulence had been established in our studies against D. melanogaster (6). In light of the findings by Ford-Siltz et al. (12) and our own results (6), the characteristics of (i) NAG formation and metabolism in diverse strains of L. plantarum, (ii) PGRP-SC expression, and (iii) larval growth and intestinal status in gnotobiotics when associated with the corresponding lactobacillus strains warrant further investigation to ascertain the regularities of the effects of the ackA gene sequence on the symbiont and host. The exceptionally high prevalence of L. plantarum strains with variant No. 2 of the ackA gene (Figure 1) underscores the significance of such studies from the perspective of the safety of bacteria with GRAS status. In light of the evidence indicating the pivotal role of bacterial metabolites, such as N-acetylated amino acids, in supporting the active growth and functioning of bacteria (15), it is imperative to devote particular attention to certain aspects. Metabolomic profiling data indicate that the "beneficial strain" of L. plantarum exhibits elevated levels of not only N-acetylglutamine, but also N-acetylglutamate and N-acetylaspartate (11). The involvement of all these compounds and their derivatives in the regulation of processes critical for adaptation of higher organisms to stressors and control of cell proliferation (including via polyamine synthesis and epigenetically mediated modulation of gene expression) (33) necessitates a comparative analysis of the relevant processes (especially the risk of malignancy) in the model organism (when it is associated with carriers of different versions of the ackA gene). This analysis should be conducted with the dual objective of clarifying the molecular scenarios of symbiosis and determining "the cost of the compromise."

3.2.4. Gut Bacteria Activate Host Stress Kinase, Which Induces Immunometabolism Remodeling

The discovery of a novel class of microbial metabolites that regulate bacterial interactions with macroorganisms has underscored the intimate link between metabolism and immunity. This finding has led to the proposal of a new concept, facultative food symbiosis, to describe the association between intestinal commensals and higher eukaryotes. A new phase in the evolution of this field was initiated by the research conducted by Grenier et al. (13). The authors of the study examined the limits of the ability of L. plantarum to

compensate for the lack of amino acids in D. melanogaster using a holadic diet. The effects of a gradual reduction in the amount of amino acids in the diet of flies, which L. plantarum is unable to synthesize, were tracked. Even under extremely harsh conditions, it was found that the association of Drosophila with the bacterium promotes larval growth. This beneficial effect is not related to providing the larvae with bacterial amino acids or stimulating their intestinal proteases with components of the lactobacillus cell wall. The authors discovered that the symbiotic bacterium L. plantarum activates the GCN2 stress kinase (General Control Nonderepressible 2) in midgut enterocytes and induces metabolic remodeling in Drosophila. This is a consequence of the GCN2 sensing of the bacterium's small RNAs, which are associated with r/tRNA. Transcriptomic analysis data indicate that L. plantarum induces remodeling of the anterior midgut of D. melanogaster. The transcriptomic profile of the studied samples revealed an enrichment with Gene Ontology (GO) clusters associated with cell differentiation and proliferation, as well as morphogenesis. In the Drosophila melanogaster model, the genes associated with organ morphogenesis, cell differentiation, and cell proliferation (especially members of the epidermal growth factor receptor [EGFR] pathway) were found to be upregulated. Conversely, the systemic growth inhibitor (Fzk) gene, genes associated with mitochondrial respiration, resistance to oxidative stress and negative regulation of cell proliferation were observed to be downregulated. In cancer cells, the phenomenon of prevalence of fermentation over mitochondrial respiration is known as the Warburg effect. The reduced expression of genes associated with mitochondrial respiration in individuals associated with L. plantarum may contribute to a specific rearrangement of host metabolism from respiration to fermentation that provides a variant of anabolism. The ability of GCN2 activation to induce a shift from respiration to fermentation has been demonstrated in mammals (34). However, the capacity of the t/rRNA sequences of L. plantarum to elicit a similar response in D. melanogaster represents a novel finding that challenges our understanding of the potential of intestinal symbionts and their arsenal of means influencing the host. The activation of GCN2 in cells of higher eukaryotes has been observed by other researchers in the study of infection of eukaryotic cells by intestinal bacteria (35). Nevertheless, this effect has been ascribed to pathogenic bacteria invading host cells. Recently, it has been demonstrated that L. plantarum is capable of penetrating and surviving within mouse and human macrophages, remodeling their metabolism, inducing a state of immunotolerance in macrophages, and developing appropriate immune memory against other Lactobacillus species (36). It remains to be seen whether lactobacilli are able to penetrate enterocytes of D. melanogaster. However, it has been demonstrated that the transfer of stress kinase activation triggers can also be carried out via extracellular vesicles of the bacterium, which have been observed to penetrate enterocytes of higher eukaryotes (14, 30, 37). The enrichment of L. plantarum extracellular vesicles with corresponding sequences of t/rRNA was demonstrated by Grenier et al. (13). In the context of intracellular infections by pathogenic bacteria, the activation of GCN2 has been linked to the potential depletion of amino acids in host cells due to their consumption by microbes. However, in the case of L. plantarum-induced activation of GCN2 in Drosophila, the effect was observed in larvae with both unbalanced and balanced nutrition. This suggests that the authors have identified a signaling mechanism for GCN2 activation that is independent of amino acid levels in the host. Instead, it appears to be dependent on the presence of bacterial molecules associated with the sequences of small RNAs (r/tRNAs) of the intestinal symbiont within the host cells. GCN2 is among the four vital stress kinases (along with PKR, PERK, and HRI, which form the core of the ISR pathway) (Figure 6). The "pan-eukaryotic" nature of GCN2, along with the protein's structural and functional conservatism, indicate its significant role in the cell signaling of eukaryotes from the earliest stages of evolution. It is evident that the involvement of GCN2 in the sensing of small RNAs from L. plantarum and the subsequent specific remodeling of host immunometabolism along the pathway of cell proliferation activation, as discovered in the studies of Grenier et al. (13), not only introduces new levels of complexity into the signaling network of this protein but also reveals new facets of bacteria with GRAS status, which are cause for concern from the perspective of biosafety. The discovery of tools in L. plantarum that can modulate host signaling systems in a manner that favors the expansion of the bacterium is both astonishing and concerning. It is astonishing to observe the evolutionarily fixed molecular mechanisms that symbiotic bacteria have developed to ensure their survival. However, it also raises concerns about potential risks associated with control of cell proliferation.. It is crucial to highlight that the data presented in this section are distinctive, as they were obtained through the utilisation of cutting-edge technologies for the sophisticated model association L. plantarum - D. melanogaster. This approach enables the tracking of causality effects, a capability that sets it apart from traditional methods. It remains to be seen whether the identified patterns are applicable to other organisms. The relevance of such an investigation is self-evident. The presented data indicate a significant limitation in our understanding of the "logic of life" of even an actively studied bacterium with GRAS status, which is widely used in practical applications. Further studies of other probiotics may yield additional insights into the molecular mechanisms of symbiosis, shedding light on the dual nature of these beneficial bacteria.

3.3. Extracellular Vesicles of Probiotics vs. Cells of Prokaryotes and Eukaryotes

3.3.1. Extracellular Vesicles of Probiotics As A Potential Alternative To Live Bacteria

It can be reasonably deduced that the most popular item in the new trend in probiotic history is related to bacterial extracellular vesicles. Extracellular vesicles (EVs) are spherical nanosized lipid particles secreted by almost every type of living cell. They serve as vehicles for a broad

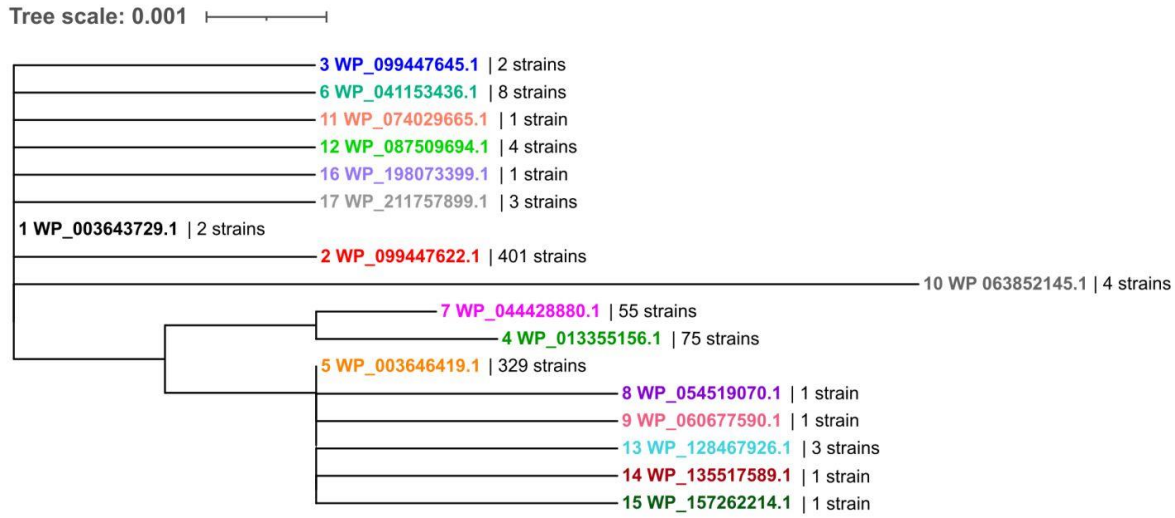

Figure 5. The phylogenetic tree of L. plantarum strains is based on the amino acid sequence of the ackA gene. The various versions of the gene (1-17) are indicated by different colors. The number of strains exhibiting the corresponding gene version is indicated on the right, according to data from the National Center for Biotechnology Information (NCBI).

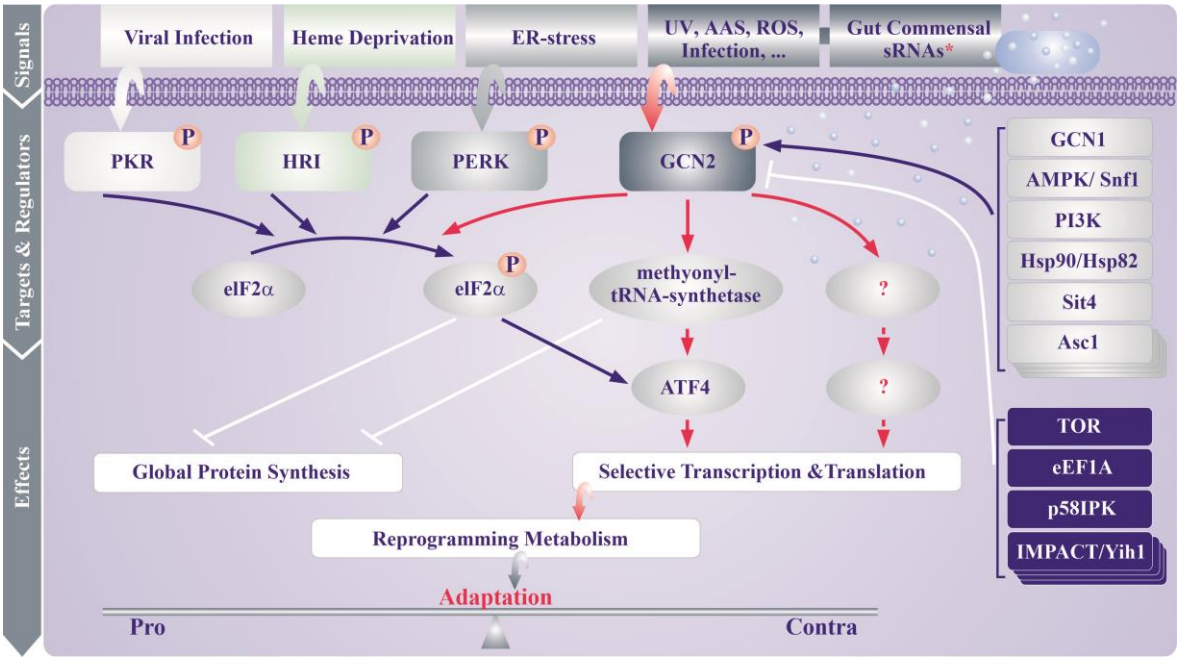

Figure 6. The role of GCN2 in integrative stress response signaling. GCN2 is one of four essential stress kinases, along with PKR, PERK, and HRI, which constitute the core of the integrated stress response (ISR) pathway. Upon activation, these kinases phosphorylate eIF2α, thereby switching translation to an economical, cap-independent pathway. This results in a change in the protein synthesis program, whereby global translation is switched to selective. Concurrently, the translation of the majority of mRNAs is repressed, except a select few, the translation of which is facilitated (among these is the transcription factor ATF4, which provides selective gene expression for stress adaptation). In contrast to other kinases, GCN2 is responsive to a diverse array of stress signals. Evidence suggests the existence of alternative GCN2 signaling pathways and potential regulatory mechanisms. It appears that transcriptomics remodeling upon GCN2 activation by L. plantarum is dependent on the presence of the bacterial r/tRNA and independent of the expression of ATF4 in the enterocytes of D. melanogaster. AMPK: AMP-activated serine/threonine protein kinase; Asc1: Activating signal co-integrator–1; ATF4: Activating transcription factor 4; eEF-1A: Eukaryotic translation elongation factor 1A; eIF-2α: Eukaryotic translation initiation factor 2 alpha; GCN1: General control nonderepressible 1 kinase; GCN2: General control nonderepressible 2 kinase; Hsp90: Heat shock protein 90. Hsp82: Heat shock protein 82; IMPACT: Impact RWD (RING finger and WD repeat-containing) domain protein; PERK: Protein kinase RNA-like ER kinase; PI3K: Phosphoinositide 3-kinase. PKR: double-stranded RNA-dependent protein kinase; p58IPK: inhibitor of PKR; ROS: reactive oxygen species; Sit4: serine/threonine-protein phosphatase PP1-1; TOR: target of rapamycin kinase; Yih1: IMPACT homolog 1. The asterisks indicate sequences of r/tRNAs derived from L. plantarum that may be transferred via bacterial extracellular vesicles. In contrast to the other three kinases, GCN2 responds to a diverse array of stress signals, triggering transcriptional reprogramming and metabolic remodeling. The dual role of this protein in immunoreactivity and tumor control represents a significant area of interest in the current scientific discourse. It has been demonstrated that GCN2 plays a role in the survival of cells, including malignant cells, by switching global translation to a selective, stress-reactive state. Additionally, GCN2 has been shown to participate in the negative regulation of cell cycle controllers, including p53 and p21 (39, 40). This has been demonstrated in cellular models, including those of mammalian and human cells, as well as in vivo, in model rodents. The potential role of small RNAs from L. plantarum, as well as other intestinal commensals and probiotics, in driving pro-oncogenic pathways via GCN2 remains to be elucidated.

spectrum of compounds, including lipids, proteins, metabolites, polysaccharides, DNA, and RNA. Additionally, they facilitate cell-to-cell communication (24, 41). Due to the lateral transport of their cargo, bacterial EVs perform a number of key functions of microorganisms, including long-distance transport of nutrients, protection from environmental stressors, intercellular communication between microorganisms and microorganisms or microorganisms and macroorganisms (hosts), and, accordingly, exert a significant influence on the outcome of the interaction (24, 41). The discovery that these nanostructures possess the beneficial properties of the original bacteria provides a rationale for exploring the potential of probiotic EVs as a substitute for live microorganisms. In 2021, the International Scientific Association of Probiotics and Prebiotics defined postbiotics as "preparations made from non-living microorganisms and/or their components that benefit the health of the host" (49). This circumstance has facilitated the exploration of potential applications for a range of novel categories of bioactive drugs, including probiotic extracellular vesicles (EVs) (24). It is presumed that the designation of Generally Recognized As Safe (GRAS) applies to probiotics and their derivatives, including postbiotics and extracellular vesicles.

The exponential growth in the number of publications on the structure and properties of probiotic extracellular vesicles (EVs) over the past decade is indicative of the profound and practical interest in these nanostructures. The findings indicate that EVs of probiotics may represent not only effective therapies for the treatment of intestinal disorders, but also a novel strategy for the development of innovative vaccines against infectious diseases and cancer, as well as the treatment of metabolic diseases such as diabetes and obesity (24). It is postulated that the utilization of probiotic EVs may prove to be a safer and, in certain instances, an even more efficacious alternative to live probiotics. Given their capacity to traverse the blood-brain barrier, EVs represent a viable means of delivering pharmaceutical agents to the central nervous system. The biocompatibility, size, and capacity to transport drugs to disparate organs and tissues render probiotic EVs a promising instrument for practical applications in biomedicine (24, 41, 42). The implementation of these promising applications necessitates comprehensive studies of the structure and functions of EVs, standardization of relevant studies, and biosafety assessments. Nevertheless, the current state of affairs in this regard is far from satisfactory. The majority of available publications do not constitute systematic studies. Rather, they are fragmented, non-standardized, and, at times, contradictory. In the overwhelming majority of cases, the focus is on demonstrating the beneficial biological effects. No evaluation of the safety of probiotic vesicles has been conducted. A comprehensive analysis of the structural and functional characteristics of probiotic vesicles, as well as an assessment of their safety, has yet to be conducted. Concurrently, distinctive data were procured for select positions in the investigation of probiotic vesicles, markedly augmenting our comprehension of symbiotic bacteria and the capabilities of probiotics.

3.3.2. Structural and Functional Characteristics of Probiotic Vesicles As A Basis for Assessing the Safety of Probiotics

Vesicle size data are regarded as a crucial element in research on probiotic extracellular vesicles (EVs). As indicated in the literature, the diameter of the vesicles is 50-300 nm and 20-200 nm in Gram-positive and Gram-negative bacteria, respectively. The results of vesicle size determination are dependent on the methodology employed. The considerable range of sizes observed for probiotic vesicles in published studies, even for a single strain of probiotic, underscores the pressing need for standardization of the isolation and analysis of EVs (24). The number of vesicles, as well as other parameters (e.g., surface charge and zeta potential), are seldom determined. Concurrently, these parameters are of significant importance for comprehending the intricate mechanisms of interaction between EVs and pro- and eukaryotic cells, as well as for the accurate design of analyses aimed at elucidating the biological activity of vesicles. The presence of a negative vesicle charge has been demonstrated to facilitate adhesion, aggregation, and the formation of biofilms (43). A significant area of focus within this field is the heterogeneity of vesicles and the representation of vesicular subpopulations. However, it is notable that this aspect has not been the subject of extensive attention to date. The identification of specific markers of subpopulations of vesicles in bacteria in general represents a separate, as yet unsolved problem. In this regard, probiotic EVs are no exception to the rule. However, the lack of resolution regarding the identification of specific markers for subpopulations of vesicles in bacteria, given their clinical significance, presents a significant challenge in the assessment of their functional potential and, consequently, the safety of these nanostructures. The assessment of the functional potential of EVs derived from probiotics in the majority of studies is associated with proteomic analysis. The total number of proteins identified in EVs from probiotics ranges from slightly over a dozen to over a thousand. However, in nearly half of the cases, these values remain within a few hundred (14,24,30,32). The considerable variability observed in the data obtained from proteomic analysis of vesicles may also be attributed to the specific characteristics of the methods employed for their isolation and analysis. The considerable range of values observed in the current literature, including those pertaining to vesicle size, underscores the pressing need for standardization of the experimental procedures. Proteomic profiling data indicates that the majority of the protein pool within probiotic vesicles is comprised of adhesins and murein hydrolases in the case of gram-negative bacteria, metabolic proteins, and PGN reorganization proteins (including murein hydrolases, lysozyme-like proteins, adhesins, and aggregation factors) in the case of gram-

positive bacteria (24). Such proteins can ensure the survival of bacteria under the stressfull conditions of host immunoreactivity and the microbial community, which trigger a number of processes, including the transformation of bacteria into L-forms, the formation and destruction of biofilms, and the suppression of competing bacteria (24, 41).. In this regard, probiotic vesicles are associated with the potential for addressing the challenges posed by negative biofilms. Recently, da Silva Barreira et al. (42) informed that their research has paved the way for the use of probiotic EVs against the development of negative biofilms. The authors identified robust antibiofilm properties of Lactobacillus casei extracellular vesicles against Salmonella enterica serovar Enteritidis (S. Enteritidis) and demonstrated that this effect is associated with two hydrolases, one of which is a protein (p40) that has been found to bind to host cells and host macromolecules (mucin, collagen), activate cell receptors, and regulate cell proliferation through the EGFR pathway. Obviously. that the practical use of these probiotic vesicles is possible only if the risks of oncogenic processes are excluded. The composition of bacterial vesicles is largely dependent on the strain-producer, the conditions of its growth, and the mode of vesicle biogenesis, which can vary even within a single strain (14, 24, 30, and 32). In the context of selective pressure, whereby bacteria adapt to stressors, particularly antimicrobials, and the profile of vesicular proteins within a given strain can undergo a notable alteration. In the case of EVs in probiotics, this phenomenon was first documented by our research group (https://repository.jpostdb.org/entry/JPST002373) as a result of studies examining the adaptation of the probiotic strain L. plantarum 8p-a3 to antibiotics (clarithromycin and amoxicillin), which was found to be associated with the evolution of virulence in the bacterium (6). It is noteworthy that in this and other instances of proteomic analysis of probiotic EVs, a considerable proportion of the vesicular proteome comprises proteins whose functions remain unknown (Figure 7). This is a consequence of the incomplete annotation of bacterial genes. It is evident that, for biosafety reasons, it is preferable to utilise only bacteria with fully annotated genomes if the bacteria are producers of EVs with potential practical applications. A noteworthy aspect of the proteome of EVs in probiotics is the presence of proteins involved in cellular metabolism. These proteins facilitate the delivery of nutrients from the external environment and can provide nutrients to both the EVs-producing microorganisms and host cells (24). Therefore, it can be concluded that EVs of probiotics contribute to the colonization of an ecological niche by bacteria and the suppression of competitors, as well as to the digestive processes of the host. In light of these observations, it appears that the vesicles of probiotics may serve as multifaceted elements of facultative symbiosis, the full functional potential of which remains to be elucidated. The cargo of vesicles is not limited to proteins; it also includes lipids, polysaccharides, metabolites, DNA, and RNA. The role of these components in bacterial survival and target reprogramming is only beginning to be revealed. Small RNAs are regarded as a crucial component of bacterial EVs, serving as a universal language for communication between prokaryotic and eukaryotic cells (44, 45).

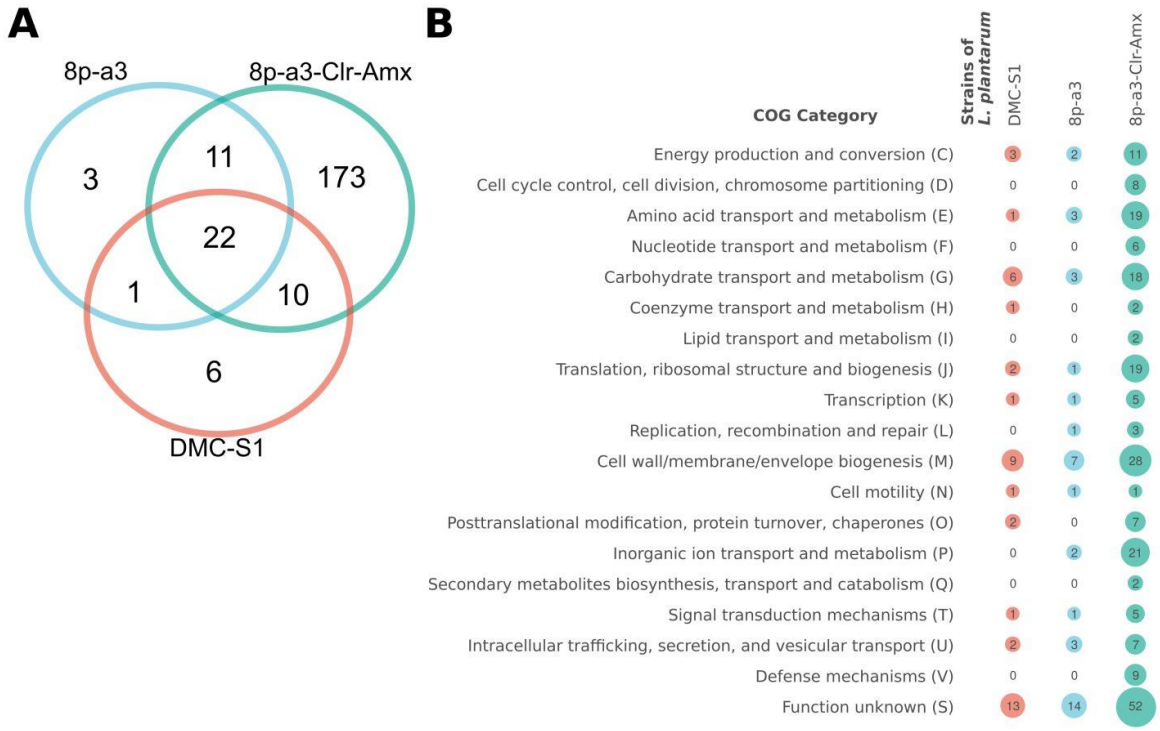

Figure 7. A Venn diagram (A) and categorization of COG (B) proteins identified in vesicles of different L. plantarum strains (8p-a3, 8p-a3-Clr-Amx, and DMC-S1) are presented.

3.3.3. Small RNAs In Probiotic Vesicles – Drivers of Target Reprogramming

The enrichment of small RNAs is a defining characteristic of vesicular cargo in a diverse range of bacterial species, as evidenced by previous research (44,45,46). This phenomenon is also observed in probiotic EVs. This was initially demonstrated by Kurata et al. (14) in the vesicles of L. plantarum JCM8341. In addition to membrane and cytoplasmic proteins, as well as metabolites, the vesicles of this strain contain a variety of small RNAs (sRNAs) associated mainly with tRNA and 5S rRNA sequences. The sRNAs present in vesicles have been linked to several key processes, including the reprogramming of pro- and eukaryotic cells, bacterial adaptation to environmental conditions, the induction of immunotolerance, and the manifestation of virulence (47). In this regard, the profile of sRNAs in bacterial vesicles and the responses of eukaryotic cells associated with the modulation of transcriptome and proteomic profiles, and reprogramming of metabolism are the focus of attention for researchers investigating the role of bacterial vesicles in intercellular communication between prokaryotes and eukaryotes, as well as the outcome of interactions between microorganisms and macroorganisms (pathogenesis/symbiosis). Bacteria have two types of small regulatory RNAs (sRNAs) that are also present in eukaryotic cells: YRNAs and tRFs (tRNA-derived fragments, tRFs). These are RNAs mapped to sequences of conserved molecules (tRNAs) that can function similarly to microRNAs (miRNAs) (44). The possibility of internalization of vesicles containing sRNAs, as well as the participation of vesicular sRNAs in RNA interference and modulation of gene expression, has been demonstrated by several works, including our own, which have examined the role of vesicles from widespread commensals relevant to medicine and biotechnology (30, 45, 48). It is postulated that the miRNA-like regulation of the expression of key immunosensors, mediated by sRNAs contained in bacterial EVs, represents a universal mechanism of interaction between bacteria and eukaryotic cells. This mechanism not only determines pathogenesis but also symbiosis. The E. coli model has recently provided evidence in support of this hypothesis. In light of these considerations, it seems reasonable to suggest that an analysis of the sRNA profile of bacterial vesicles and the associated gene expression responses in eukaryotic cells should form an integral part of the assessment of pathogenic potential and the safety of vesicles intended for practical applications. This is of great consequence for probiotic EVs, as recently demonstrated by Yu et al. (30). The authors identified sRNAs contained in the vesicles of L. plantarum WCFS1 and demonstrated that the strain has the potential to suppress the expression of the p53 protein, which serves as the "guardian of the genome," the controller of genomic stability, and the tumor suppressor. This capacity is conferred by the presence of a particular sRNA, which can be conveyed through the extracellular vesicles of the bacterium, transported into human cells, and suppress the expression of the p53 protein gene. The evolutionary significance of the presence of the corresponding molecule in the vesicles of a strain of widely distributed commensal bacteria is of considerable interest to fundamental researchers of the "logic of life" of these microorganisms. However, this phenomenon gives rise to acute questions for practitioners, the first of which is the prevalence of the corresponding property in the vesicles of different strains of L. plantarum. It is noteworthy that the aforementioned study by Kurata et al. (14), which characterizes the cargo of EVs from a different strain of L. plantarum (JCM8341), lacks data indicating the enrichment of the vesicular pool of sRNAs with a molecule that is homologous to the sequence of the p53 gene. However, it is noteworthy that the vesicular sRNAs appear to be enriched with sequences associated with tRNA and 5S rRNA. This suggests that vesicle composition in different strains of L. plantarum varies in terms of both quantitative and qualitative characteristics of the respective sRNA classes. It would be of great interest to ascertain whether the vesicles of L. plantarum strains are enriched with rRNA and tRNA sequences that trigger the activation of the GNC2 stress kinase (13). Furthermore, it would be beneficial to determine the characteristics of the prevalence of strains whose vesicles are enriched with sRNA homologous to the sequence of the p53 gene. Additionally, it would be advantageous to investigate whether the above scenarios are realized in vivo, as well as how these aspects are performed in other probiotics. These questions are of great relevance to biosafety. As previously stated, the GNC2 kinase plays a role in the negative regulation of the key controllers of the cell cycle and proliferation, namely p53 and p21 (39, 40). In light of these considerations, the practical applications of probiotic vesicles (even if they lack the target sRNA that suppresses p53 expression but are enriched with sRNAs associated with rRNA and tRNA sequences) give rise to concerns about the pro-oncogenic potential of such nanostructures. To ascertain the pertinent risks, a comprehensive examination of the vesicle content of all probiotic strains employed and the clarification of the molecular mechanisms underlying their interaction with pro- and eukaryotic cells in cellulo and in vivo contexts is imperative. Such research should be a priority shortly.

3.3.4. Extracellular Probiotic Vesicles for Practical Applications: Problems And Prospect

The dialogue between bacteria and eukaryotic cells is carried out through EVs, and there is undoubted progress in the study of its machinery. However, there are also significant gaps in knowledge that must be addressed to fully comprehend the principles of interaction between micro- and macroorganisms, the survival of bacteria under selective pressure, the evolution of their virulence, and the molecular machinery of symbiosis. These include the following areas of research: (i) the mechanisms underlying the biogenesis of bacterial EVs and the sorting of cargo; (ii) the role of epigenetic factors in EVs and the mechanisms of EVs-mediated immunotolerance; (iii) the mechanisms of

EVs-mediated modulation of the gut microbiome; and (iv) the mechanisms of EVs-mediated modulation of the tumor microenvironment; (v) patterns of changes in the composition of EVs and the outcome of cross-talk between pro- and eukaryotic cells (pathogenesis/symbiosis) (24, 41, 47). With regard to probiotic EVs, it is of great importance to ascertain these questions in order to identify all the positive and negative aspects of the bacteria. The primary challenge in vesicle research is the lack of standardization. In this regard, the available data on the beneficial biological activity of probiotic EVs (such as anti-inflammatory and immunostimulating, immunosuppressive and antiproliferative, geroprotective and antidepressant effects, improving the structure of the microbiota and reducing intestinal permeability (24)) revealed in cellulo (using various mammalian and human cell lines) as well in vivo (using rodents such as mice and rats as animal models (46, 47)) require caution in their interpretation and conclusion about the suitability of the relevant nanostructures for practical application. To obtain accurate information about the structure and biological activity of EVs, it is essential to adopt an interdisciplinary approach that employs a range of high-resolution techniques, including modern post-genomic technologies. Furthermore, it is crucial to standardize the research methodology to ensure the reliability and reproducibility of findings. Until these issues are resolved, the problem of assessing the safety of microorganisms widely used in medicine, agriculture, and biotechnology remains unresolved.

Conclusion

Intestinal commensals, bacteria with GRAS status, and probiotics represent an integral component of the prokaryotic kingdom, and as such, they possess a robust, evolutionarily conserved system of self-preservation. This system enables bacteria to survive in a hostile environment by acting in their own interests, including by suppressing the life support systems of competitors and/or the host organism. As new technological avenues for scientific research have opened up in the 21st century and new knowledge has emerged about the "dark" and "light" sides of microorganisms, it has become evident that some of the long-held beliefs regarding the exceptional benefits of probiotics must be reconsidered. A substantial contribution to this comprehension has been made by the data of a multitude of meta-analyses on the associativity of effects, in addition to the findings of fundamental studies in vitro, in cellulo, and in vivo conducted on model systems that facilitate the discernment of causality. The application of cutting-edge high-resolution techniques, including post-genomic methodologies, has led to the acquisition of data that has profoundly impacted our comprehension of commensals, symbionts, and probiotics, evoking a range of emotions, including surprise, delight, and trepidation. The present review offers a first account of the analysis of the properties of probiotics that may pose a threat to their safety. The new knowledge yielded an unexpectedly important result: the reaffirmation of the old truth, "Sola dosis facit venenum." It has been demonstrated that the microbial load within the gut is of consequence, even when the bacteria in question are beneficial. The search for the "optimal" dose represents a novel challenge that will require further investigation in the future. The reports of phenomenally high levels of genomic plasticity, the possibility of large-scale genomic reorganization, and the progressive evolution of virulence in these bacteria under the conditions of selective antimicrobial pressure, the presence of unique tools for expansion, suppression of host immunoreactivity, modulation of the gut microbiome, negative regulation of key cell cycle controllers, and activation of host cell proliferation indicate a clear insufficiency of our knowledge regarding the "logic of life" of "beneficial bacteria." This insufficiency compromises popular practical applications. A significant piece of relevant data was obtained for L. plantarum, a bacterium with GRAS status, strains of which are widely used as probiotics. Additionally, the association of this bacterium with D. melanogaster, a classic model organism for which L. plantarum is considered an intestinal symbiont, was established. Concerning other organisms, there are no systematic studies, only fragmentary ones. The alarming facts of some of these studies indicate a need for similar studies on other organisms. This is relevant for two purposes: (i) elucidating the diversity and patterns of probiotic effects that can be beneficial or dangerous to human, animal, and environmental health, and (ii) developing a global system for the control of probiotics. Clearly, this necessitates comprehensive studies of probiotic bacteria based on standardized protocols for safety and toxicity, as well as the consolidation of the efforts of medical professionals, biologists, physicists, chemists, and toxicologists. It is evident that scientific knowledge has the potential to facilitate breakthrough solutions and expose dangerous misconceptions. However, it is also clear that the will of regulators is necessary to overcome the spread of such misconceptions.

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Authors' Contribution

Concept and design of the study: C.OA.
Drafting of the manuscript: C.VM, C.OA.
Statistical analysis: M.MI.
Administrative, technical, and material support: T.MV, C.VM.

Ethics
We hereby affirm that all ethical standards have been observed in the preparation of the submitted article.

Conflict of Interest
The authors declare that they have no conflict of interest.

Data Availability
All bacterial genomes cited in this article are publicly available on the NCBI website (https://www.ncbi.nlm.nih.gov/datasets/genome/).

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