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Omics in Animal Nutrition Research

Key information

Many developments in the field of animal and nutrition research have occurred in recent years. One major change is the use of Omics techniques to investigate the effects of challenges and treatments in the animal on a broad molecular level (Beauclercq *et al.*, 2016; Beauclercq *et al.*, 2018; Le Roy *et al.*, 2016; Li *et al.*, 2012; Pérez-Bonilla *et al.*, 2018). Whilst these new types of analyses allow a much deeper systematic insight into the biology of animals and their interaction with nutrition, they also bring new challenges. The aim of this review is to describe the basic principles of Omics techniques, and to help the reader understand the opportunities and limitations of these techniques.

What is Omics?

Omics is a term used to refer to a specific set of analytical techniques used to measure biological samples. Omics techniques aim at measuring the total composition of a specific biochemical group: DNAs, RNAs, proteins, small hydrophilic compounds, and small lipophilic compounds. The different types of Omics reflect the different levels of molecular biology, namely the genome (DNAs), transcriptome (RNAs), proteome (proteins), metabolome (hydrophilic metabolites), and lipidome (lipophilic metabolites) respectively. So, proteomics aims at measuring the chemical composition of all proteins, transcriptomics at measuring all RNA molecules in a given sample etc.

Although these methods are not able to truly cover all entities of their respective chemical group, they cover a much broader range of chemical diversity in biological samples in comparison to older methods of quantification. For example, in animal nutrition research typically only the 20 proteogenic amino acids are quantified in a plasma sample, but with metabolomics the quantification of up to 1000 different metabolites is possible. Older methods target a few specific entities for quantification, whereas Omics methods try to cover as many entities as possible and are thus also called untargeted methods of quantification.

Omics techniques therefore allow for a more in depth understanding of the system as a whole because within a biological level a researcher can assess the state of all molecules in the respective system. Multiple Omics techniques can be used in the same study to investigate how different levels of molecular biology are connected and influence each other. A specific field called Systems Biology has developed which often combines multiple Omics tools and specific trial designs to further the understanding of whole biological systems.

Where and why is Omics used?

Omics have been used in animal and nutrition research for many years. When the techniques were new and less established, more fundamental questions regarding the regulation of growth in muscle and its connection to other vital organs like liver were the focus of investigations (Carkett and Logan, 2011; Hazard *et al.*, 2011). These studies led to an understanding of the key molecular processes involved in growth of farm animals and how feed

or other environmental factors influence these growth pathways. Likely, many of these insights were used to define genetic targets for the superior growth performance of modern livestock. From the beginning there was an additional focus on understanding disease mechanisms including how pathogens or treatments achieve their effects on a molecular level (Burgess, 2004; Reemers *et al.*, 2010). For example, Pérez-Bonilla *et al.* (2018) used metabolomics of chicken breast samples to investigate molecular causes of white stripping and found that hypoxia induced changes in the energy metabolism and nitic oxide might be at fault. Recently, application-driven research is thriving and with it another focus has appeared: microbiome analysis using metagenomics techniques (Han *et al.*, 2017; Oakley *et al.*, 2014; Whelan *et al.*, 2019). Here researchers investigate different causes of deteriorating gut health and the impact of respective gut health solution products. All in all, most commonly Omics methods are used in animal sciences to investigate the molecular causes of a particular phenotype.

Basics of analytical methods in Omics

The physiochemical properties of the entities of interest determine the analytical method used. DNA and RNA have a low chemical complexity and diversity (Figure 1). They consist of four nucleic bases arranged on a phosphate-sugar backbone with a consistent structure. These nucleic acid molecules can be large and are relatively stable in an aqueous environment, which is especially true for DNA. While RNA molecules can be degradation sensitive, simple biochemical methods easily transcribe RNA into stable DNA without any loss of information. Thus, sequencing is normally performed with DNA molecules and RNA is transcribed into DNA before sequencing for transcriptomics. Proteins and small molecule metabolites on the other hand have a high chemical complexity and diversity. Their size and stability are highly variable, ranging from the biggest protein to the smallest molecule. The variability in chemical stability makes analyzing a lot of different proteins and small molecules at once especially difficult. Additionally, unstable molecules can degrade into stable ones after the extraction from the sample and thus change the chemical composition of the original sample.

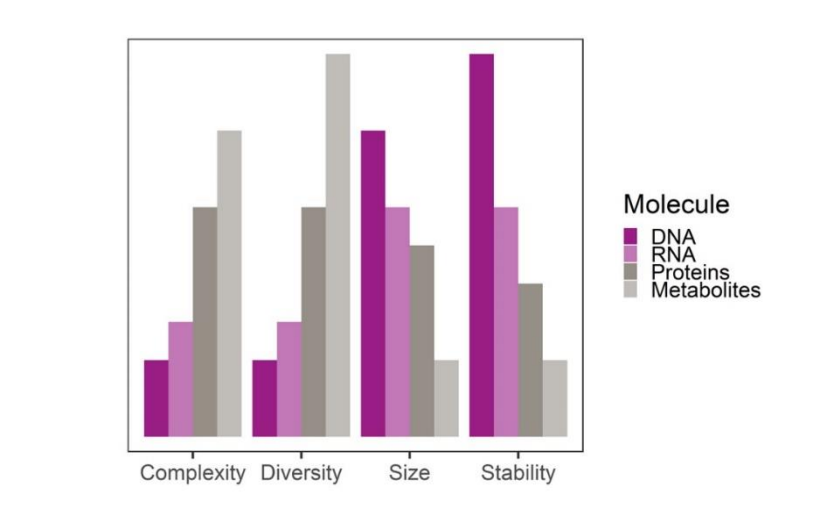

Figure 1: Chemical properties of biological molecules. Chemical properties of the molecules determine the method and technique used for their analysis.

In consequence, there is a basic split in Omics between the technologies used to investigate DNA and RNA and those used to investigate proteins and small molecules. Sequencing methods are used to analyze DNA and RNA, which has been transcribed into DNA before analysis. Methods for analyzing DNA often have sequencing or seq in their name, e.g. RNASeq or 16S Sequencing. Sequencing methods have matured in recent years, and the price to determine the sequence of DNA has dropped exponentially. Conversely, mass spectrometry and nuclear magnetic resonance (NMR) are the methods used to analyze proteins or small molecules. Complex abbreviations are often indicative of methods for proteins or small molecules like LC-MS/MS or MALDI-MS/MS. Nowadays, mass spectrometry is used often over NMR as it is more sensitive and allows an easier automation for high

throughput experiments. The chemical complexity of the proteomic or metabolomic analytes makes automated analysis hard but many new developments in different areas of the field improve the analytical capacity steadily. As such the prices for metabolomics or proteomics analysis are also dropping although due to the technical complexity not as rapidly as for sequencing.

Principles in animal sciences also apply with Omics methods

As seen from the examples above, Omics is not a science in and of itself; rather, it is a set of methods used to investigate the experimental subjects in specific research questions. As such, the same scientific rigor applied to other method of analysis should be applied to Omics as well. Experimental design, choice of treatment and measurement parameters are key for the success of any experiment, including those using Omics methods. Data quality checks need to verify that experimental data meets key assumptions. In a feeding trial an abnormally high feed conversion ratio may indicate a faulty experimental method or outliers caused by errors in sampling or mislabeling. Likewise, Omics have key assumptions that must be met to determine data quality. For example, liver tissue and muscle tissue should show distinctive gene expression in transcriptomics as we know these tissues look and function differently. Likewise, the blood metabolome of animals fed methionine-deficient diets should differ from those fed methionine-adequate diets in the concentration of methionine related metabolites. When data quality checks are passed successfully, rigorous use of applicable statistical methods is as important as in any other kind of scientific experiment. Finally, interpretation of results needs to carefully assess all available data and stay in context of the tested experimental design.

In comparison to classical analytical methods, visualization of data and results with graphs is an essential step of any Omics data analysis. Because Omics method measure up to 40,000 different molecules per sample, impossible to be processed by a human mind alone, descriptive data visualization is crucial and guides the understanding of observed effects. Data analysts typically use similar approaches and figures, three of which will be explained in context of their intended descriptive goal. These figures are by no means the only ones used, but commonly at least one can be found in any Omics research paper.

What looks different, is different

As Omics methods measure the molecules which make up the cells in a biological sample, visual and other known differences between the samples can help with quality control and interpretation of the results. The expected differences between should be reflected in the results of the Omics analysis. For example, swine liver tissue is brown and swine muscle tissue is pink. These visual differences are caused by different small molecules in the tissue, which are caused by different enzymes being present, which are caused by differences in gene expression. Thus, the results from metabolomics, proteomics or transcriptomics of liver tissue should reveal that many metabolites, proteins or RNAs respectively are different between the two. Indeed, the molecular composition of liver tissue is different than the composition of muscle tissue (Vigors *et al.*, 2019)). The same holds true for small variations. If the treatment of interest does not induce visible differences in the animal, it is unlikely that many molecules are affected. Therefore, it is unlikely strong differences will be observed with Omics methods. For example, given everything else is similar, healthy animals from different farms cannot be visually distinguished from each other, and as such also the differences which could be found with Omics methods are small (Vigors *et al.*, 2019). However, growth performance effects not stemming from disease are harder to classify: intermediate effects on the swine muscle transcriptome were found (Vigors *et al.*, 2019), but only minor effects on the broiler blood metabolome (Hofmann *et al.*, 2019). As growth is a complex trait affected by many factors, Omics effects vary depending on the reason for the difference in growth. Thus, depending on experimental design and Omics method, we can have different expectations about the number of molecules which should change. For example, if performance is limited by only one nutrient, effects on the blood metabolome can be minor in comparison to effects induced by diets comprised of different feed stuffs which do not affect performance (Hofmann *et al.*, 2019). Thus, if the treatment is not a matter of molecular diversity it is less

obviously picked up by Omics methods. Consequently, when making assumptions about Omics measurements we should ask ourselves: For the respective molecular biological level, does the treatment likely affect very few molecules or does it affect many different ones?

In conclusion, if phenotype differences are small, molecular differences are small; conversely, if differences in phenotype are large molecular differences are large as well. The benefit of Omics technology is that it allows us to understand what is changing on a deep molecular level to cause the variance in phenotype, regardless of the magnitude of that change.

Visualization of diversity

To investigate how different or similar samples are from each other, researcher often use multidimensional scaling methods, e.g. perform a principal component analysis (PCA) or principal coordinates analysis (PCoA). Essentially, these statistical methods visualize the level and direction of dissimilarity of individual samples within one dataset based on all Omics measurements for those samples. The first principal component accounts for as much of the variability in the data as possible, and each succeeding component in turn has the highest variability possible under the constraint that the origin of variability has not been captured by the preceding components.

For example, Figure 2 shows a PCA based on a transcriptomics dataset from Vigors *et al.* The first PC carries 82 %of the total variability of the transcriptomics dataset and the second PC caries only about 6 %. Like meter and millimeter as units for distances these percentages tell us the scale of dissimilarity between samples. Sample A has on paper the same distance to Sample B than to Sample C. However, the scales on the x- and y-axes tell us that Sample A is much more different from Sample B than from Sample C, just like 1 m is much farther than 1 mm. Depending on the method used for the calculation of dissimilarity, this can mean that many genes are slightly differently expressed between the samples, or that some genes are very strongly differentially expressed between the samples.

Multi-dimensional scaling procedures are unsupervised statistical methods, so knowledge about sample groups is only added after the calculation. The researcher can visualize if samples separate according to treatment or if other key factors also have an effect. Figures 2 to 4 show the same PCA based on the transcriptomics dataset from Vigors *et al.* but the samples are colored according to different sample properties. We see that liver and muscle tissue have very different gene expression (Figure 2), whereas farm of origin does not affect gene expression a systematic way (Figure 3). When coloring according to growth performance (Figure 4) we see that muscle tissue from high and low performing pigs have moderately different gene expression whereas in liver tissue these differences cannot be found. This does not mean that there are no performance-based gene expression differences in liver tissue, just that in comparison to muscle tissue the differences are a) much smaller if any and b) not in the same genes as in the muscle tissue. We might find an effect on liver if we look at additional PCs, e.g. the next in order PC3 and PC4.

In summary, a PCA or PCoA allows researchers to see in one unbiased glance which experimental factors affect the Omics measurements the most. Further analysis of the data can then focus on the most relevant treatment comparisons and investigate exactly which molecules are affected by the treatment.

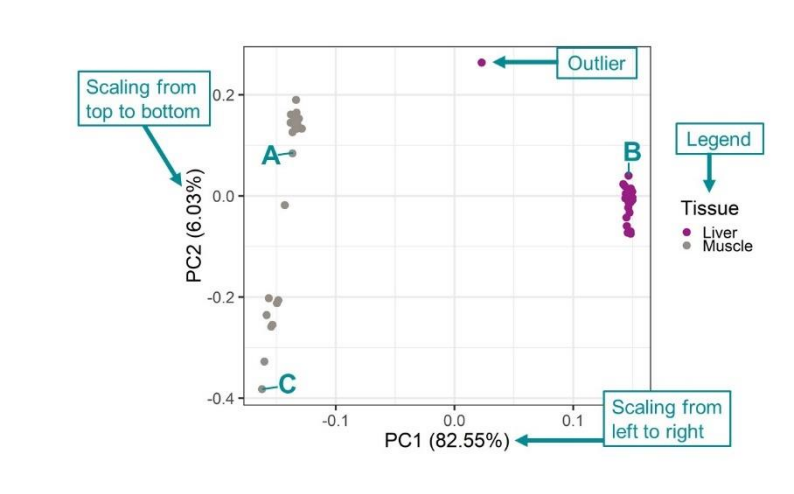

Figure 2: PCA of swine liver and muscle tissue transcriptomics. Color according to tissue type. Adapted from (Vigors *et al.*, 2019).

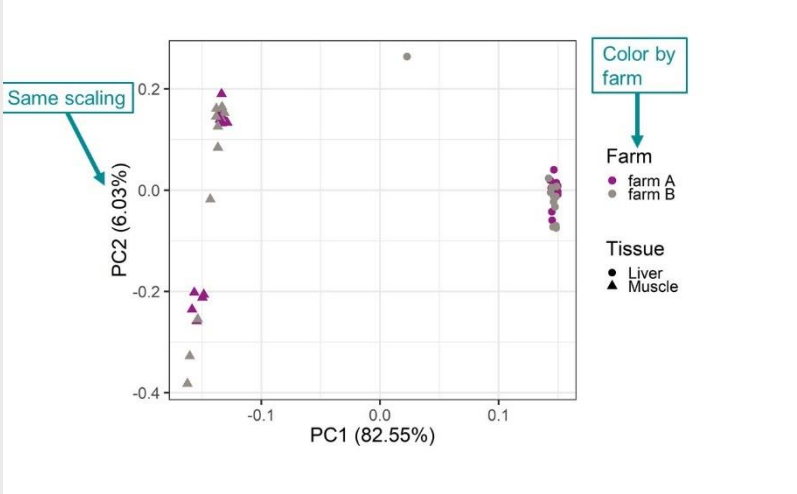

Figure 3: PCA of swine liver and muscle tissue transcriptomics. Color according to farm of origin, shape according to tissue type. Adapted from (Vigors *et al.*, 2019).

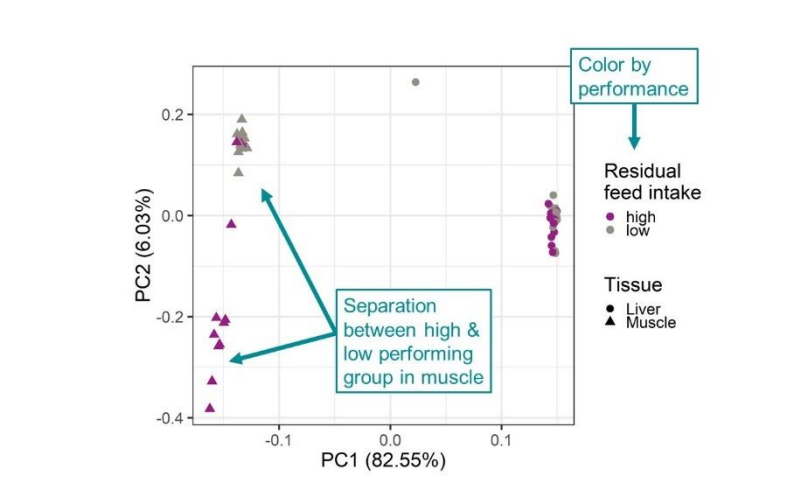

Figure 4: PCA of swine liver and muscle tissue transcriptomics. Color according to growth performance, shape according to tissue type. Adapted from (Vigors *et al.*, 2019).

Everything Omics is relative

There are important points which are hallmarks of all Omics methods and which make them different from other types of measurements. The first one was already discussed: In contrast to targeted analytics, Omics methods are usually untargeted and aim to measure the total composition of a specific chemical group, e.g. metabolomics quantifies the composition of most metabolites in a given sample. As the main goal of Omics is to measure diversity of chemical composition, the ability to measure many molecules at once is traded off with the ability to measure the absolute concentration of those molecules. This does not mean that sequencing or mass-spectrometry based methods are per se unable to measure absolute quantities of a single targeted molecule. However, absolute quantification requires additional resources which cannot be applied when investigating a diverse set of molecules at once. The consequence is that the values for each molecule in Omics are on an arbitrary relative scale and that scale is not comparable between different molecules. It is like knowing your supplier is delivering 5 items but not knowing if that means *single* apples or *buckets* of apples. Further, just because one now knows it means *buckets* of apples it does not mean the same scale is true for deliveries of watermelons. However, 10 items are double the amount of 5 items in both cases. Thus, it is possible to compare the abundance of a specific gene between different samples (all on the same gene-specific scale) but not between different genes from the same sample (different gene-specific scales). The specific reason for this diversity-absolute quantification trade-off is different between sequencing methods and mass spectrometry-based methods. The consequence is the same: Instead of comparing absolute numbers like mg/L between different samples, relative comparisons like 2-times higher or in scientific terms a foldchange of 2 are used.

Visualization of relative expression

Typically, researchers use one of two different ways to visualize statistically relevant results from an Omics experiment: Volcano plots and heatmaps. Both emphasize the relative nature of Omics data and both restrict the data to visualize what is relevant and significant. Volcano plots show only the mean fold change between two treatments for every gene instead of data points from every sample, and heatmaps show only significantly affected genes for every sample instead of all genes.

In a volcano plot (Figure 5) each dot represents an Omics entity, in this case a gene. The data is typically shown using logarithmic scales in order to easily visualize all genes in a single figure despite large differences in expression or statistical significance. The x-axis shows the relative change in expression – the fold change - for that gene between high and low performing experimental groups on a log2 scale. This means dots on the very right of the plot represent genes which have 2^2 or 4 times higher expression in high performing pigs than in low performing pigs. The y-axis shows the p-value of a statistical test on a negative log10 scale, meaning dots on the very top of the plot have a very low p-value of 0.1^5 or 0.00001. The dots are color coded according to whether they are statistically significant between treatments (using the common threshold of $P < 0.05$). Thus, the most interesting genes end up on the top right or top left corner highlighted in purple. A researcher can then decide to investigate those genes more closely for their function and relevance to answer the experimental question.

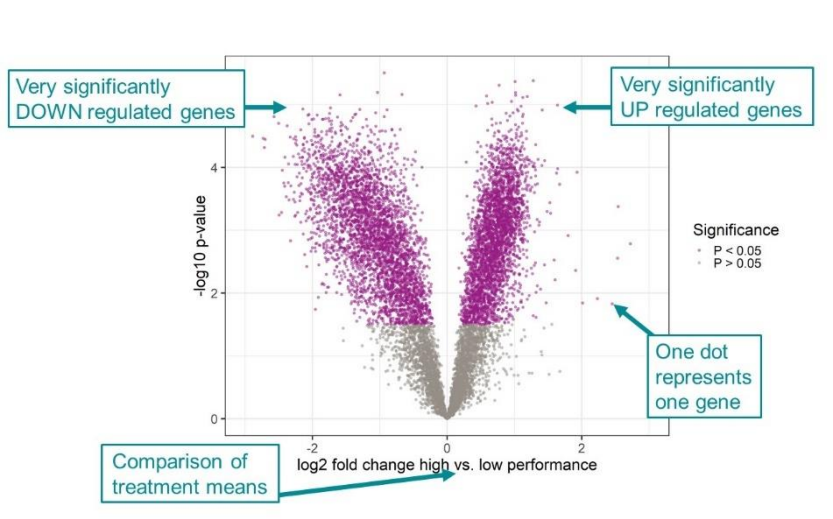

Figure 5: Volcano plot of swine muscle tissue transcriptomics results. Color according to significance: BH method multiple testing corrected p-value from 2-way ANOVA with performance and farm of origin as factor. Adapted from (Vigors *et al.*, 2019).

Using the same data for a heatmap (Figure 6) lets the researcher investigate a different pattern. In a heatmap each row is representing one significantly differentially expressed gene, and each column represents a sample. The purple and black squares on top of the columns indicate which treatment the samples belong to. The fold change of each gene is indicated by the color in the heatmap, blue indicating lower expression in that sample than on average across all samples and red indicating higher expression than the average. Often the data in heatmaps is clustered in a dendrogram to visualize which samples behave similarly across their gene expression. Alternatively, clustering can be used on rows as well, indicating in this case which genes behave similar across all samples. The dendrogram shows how those clusters are similar or different to other clusters. In this example we can see how the purple squares generally cluster more closely to other purple squares and black to black. We also see that lighter colors do not cluster well within the big clusters. This tells us that muscles from high performing pigs (purple) behave similarly, and differently compared to muscles from low performing pigs (black). Additionally, we see that farm of origin still does not play a role when only taking significant genes into consideration. This data representation allows the human eye to quickly grasp which samples and which genes behave similarly and to assess the uniformity of gene expression across samples of the same group. This also allows a researcher to select a specifically interesting gene cluster distinguishing the sample groups from each other for further investigation. The genes in that cluster may indicate that a specific molecular pathway is activated, which leads to a hypothesis explaining why the samples in the different groups behave different.

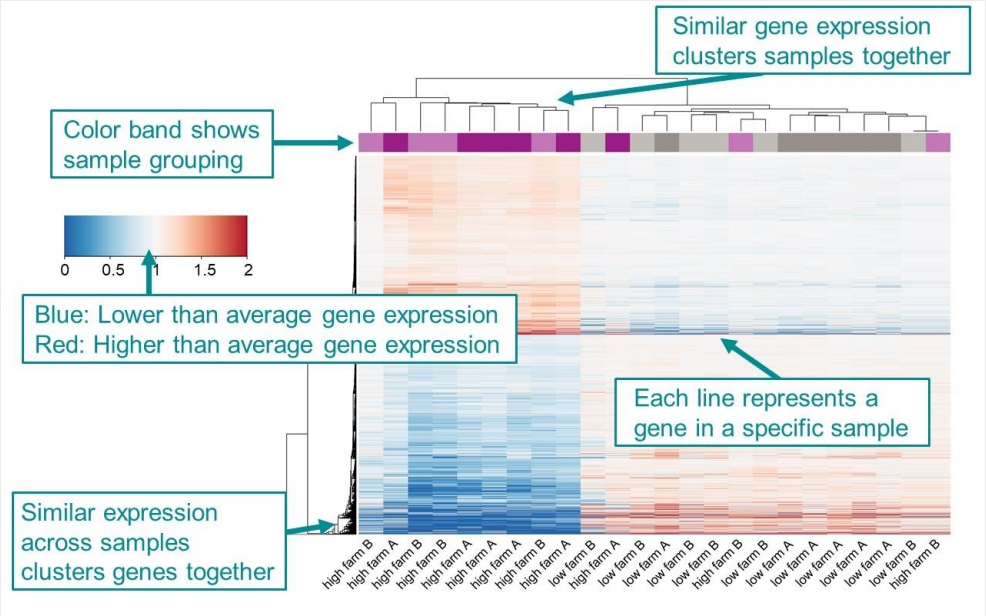

Figure 6: Heatmap of swine muscle tissue transcriptomics results. Only genes significantly changed between low and high performing pigs (2-way ANOVA, BH corrected, $P < 0.05$) are part of this heatmap. Dendrograms using Complete clustering method of Euclidian distances. Adapted from (Vigors *et al.*, 2019).

Omics is not as sensitive or precise as targeted methods

Another distinguishing trade-off for many Omics methods besides the relative molecule specific scale is that they are not as sensitive or as precise as targeted approaches. In favor of measuring chemical compositional diversity the methods cannot be optimized to achieve high sensitivity and selectivity for each possible molecule. Targeted methods like qPCR for gene-specific measurements reach sensitivity of 4 gene copies/sample whereas typical transcriptomics methods quantify in ranges of 100,000 gene copies/samples (Owens *et al.*, 2016). Targeted and untargeted mass spectrometry methods for metabolite measurements both quantify molecules with a concentration of less than 1 ng/mL respectively. However, untargeted methods have a much higher sample-to-sample variation than targeted methods with higher chances of randomly missing a metabolite despite it being present in the sample (Do *et al.*, 2018). So, while Omics methods give an idea about big picture changes in whole biological system under investigation, they are not the best choice to show how a very specific part of the system changes. As such, Omics is often used to generate a hypothesis about a biological response, but targeted methods are then needed to validate a specific underlying mechanism.

No gene is an island: Function and context are key for understanding

The result of Omics experiments is usually a difference in abundance between control and treated samples for a few of the thousands of measured molecules. For example, the transcriptomics experiment in pigs resulted in finding ~6500 out of ~12600 measured genes differently expressed between muscle-tissue from high performing or low performing pigs (Vigors *et al.*, 2019). We can visualize these differences with Volcano plots and heat maps (Figure 5, 6) and make a list of which genes are affected. While listing the names of all those genes will not help us to understand what is going on in the tissue, categorizing the function of those genes will. Researchers categorize genes and other molecules according to an associated function previously described in the scientific literature by other researchers. These classifications are collected in databases like Gene Ontology (GO) (Ashburner *et al.*, 2000) or KEGG metabolic pathways (Kanehisa and Goto, 2000). Through decades of molecular biological research, it became obvious that many genes are needed to fulfill one function and that one gene can have many different functions depending on the context it is expressed in. For example, immune pathways and muscle growth pathways share many genes because a key function of both muscle cells and immune cells is to replicate and grow (Figure 7). So, an association with immune pathways is often found in growing tissues not

necessarily because the tissues are inflamed or the immune system is activated but because other researchers found that the same genes associated with growth in muscles tissue are also associated with immune cells replicating when activated. The same is true for the swine muscle experiment from Vigors: The affected genes are involved in RNA processing, and many RNA processes like ribosome biogenesis and tRNA processing likely involve partially the same genes (Figure 8). These many-to-many relationships make interpretation of the data difficult, but with careful consideration of the biological context, e.g. which tissue type was sampled, which treatment administered, or if some of the other 400 immune genes not associated with growth are affected, these pathway associations can guide the researcher to useful testable hypotheses about the inner working of the investigated tissues.

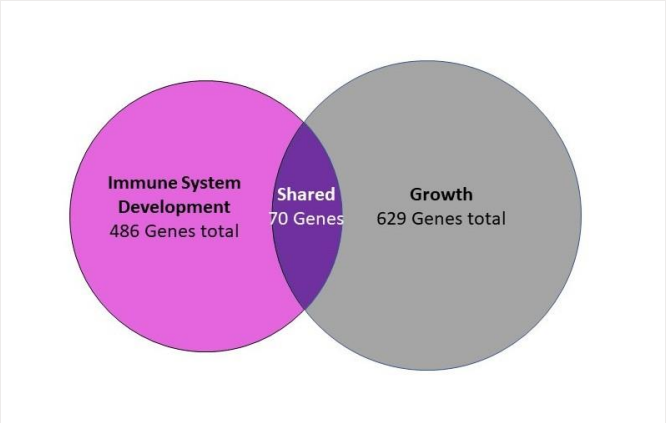

Figure 7: Venn Diagram showing overlap of genes between two GO terms. For Gallus gallus 70 genes out of 486 which are associated with GO term “Immune System Development” are also associated with GO term “Growth”.

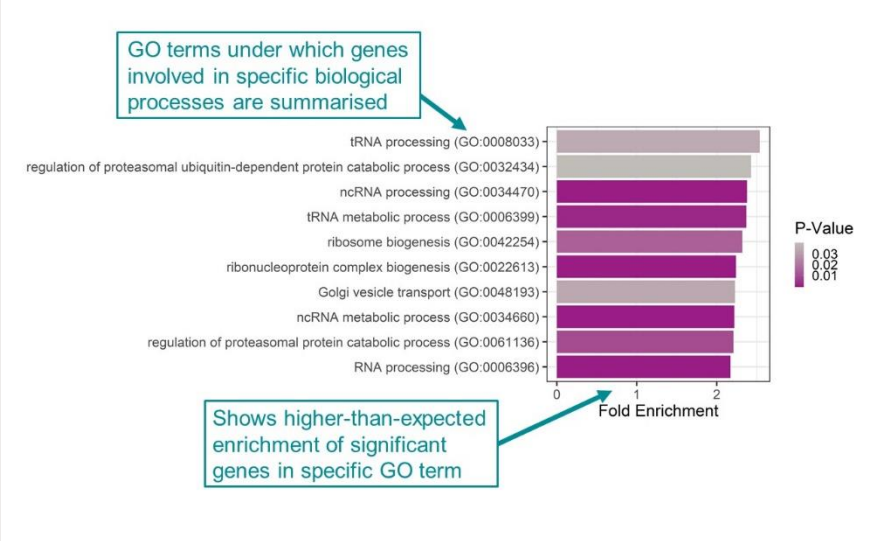

Figure 8: GO term enrichment of swine muscle tissue transcriptomics results. Enrichment (Chi-square test, $P < 0.05$) of genes significantly changed between low and high performing pigs (2-way ANOVA, BH corrected, $P < 0.05$) in respective GO terms. Adapted from (Vigors *et al.*, 2019).

Trust, but verify: test the hypothesis

As Omics methods estimate molecular diversity but cannot quantify on a sensitive absolute scale, they are useful for the description of a system but do not confirm its function. It is like describing a car in all its complexity and being able to compare its features to other cars. In the end in order to prove it is working like you think it is you will have to start the engine and drive. Thus, Omics analyses should be accompanied by or followed up with functional analyses supporting the hypothesis derived from the Omics data. For example, a transcriptomics analysis of liver tissue shows upregulation of immune system related genes in animals treated with a specific drug

versus non-treated animals. One might want to conclude that the treated animals have better immune function. However, a change in expression of immune regulation genes might instead actually be indicative of a worse immune response. Alternatively, as previously described these genes might indicate no change in the status of the immune system at all but rather due to changes in muscle cell growth. Without additional evaluation of the actual functional immune response of the animals in terms of faster vaccine response or faster recovery from challenge there is no proof for either of these conclusions. However, now the researcher has a specific research question at hand to investigate the molecular function of the drug and might find a phenotypical outcome associated with it, whereas before there was no indication of the drug having any effect or where to look for that effect. In conclusion, Omics methods cannot replace phenotypic observations like better feed conversion ratio or faster vaccination response, but they can guide the researcher towards a targeted testable hypothesis about the underlying molecular mechanism behind it.

Summary

Omics is not a field of science in itself. Rather, it is a set of methods used to measure experimental subjects in specific research questions. At their core, Omics techniques aim at measuring the total composition of a specific biochemical group: (meta)genomics for DNAs, transcriptomics for RNAs, proteomics for proteins, metabolomics for small hydrophilic compounds, and lipidomics for small lipophilic compounds. The physiochemical properties of the chemical entities of interest determine the analytical method used. Therefore, there is a split in Omics between sequencing-based methods for DNA and RNA and mostly mass-spectrometry based methods for proteins, metabolites and lipids. In all methods however, the ability to measure many molecules at once is traded off with the ability to measure the absolute concentration of those molecules. Additional trade-offs are a limited sensitivity for sequenced-based methods, and a lower precision for mass-spectrometry based methods in comparison to their targeted counterparts.

Notably, due to the amount of data generated, in comparison to classical analytical methods, visualization of data and results with graphs is an essential step of any Omics data analysis. Usually, PCAs or PCoAs are utilized to investigate general differences between samples and show the effect of experimental treatment on the Omics entity under investigation. For statistically significant effects heatmaps or volcano plots are useful to guide the selection of interesting molecular patterns. Furthermore, researchers categorize the genes (and other molecules) according to an associated function, which was previously described by other researchers and collected in databases. Often one molecule is involved in many processes, and one process is comprised of many molecules. These many-to-many relationships make interpretation of the data difficult, but with careful consideration of the biological context these pathway associations can guide the researcher to a useful testable hypothesis about the inner working of the investigated samples.

The fundamental achievement of Omics is that a phenotypic observation in an animal trial can be combined with measurements of most molecules which make up the biological environment in a sample (Figure 9). Thus, visual and other known differences between the samples can support the interpretation of Omics results and lead to a conclusion which explain the molecular mechanism causing the phenotype. However, while Omics methods give an idea about how the whole biological system under investigation changes, it is not the best choice to show how a very specific part of the system changes. Thus, targeted methods or a targeted new experiment need to complement trials with Omics methods to test the hypotheses from the Omics results. Omics methods cannot replace a phenotypic finding like better feed conversion ratio or faster vaccination response, but they can guide the researcher towards a targeted testable hypothesis about the underlying molecular mechanism behind it.

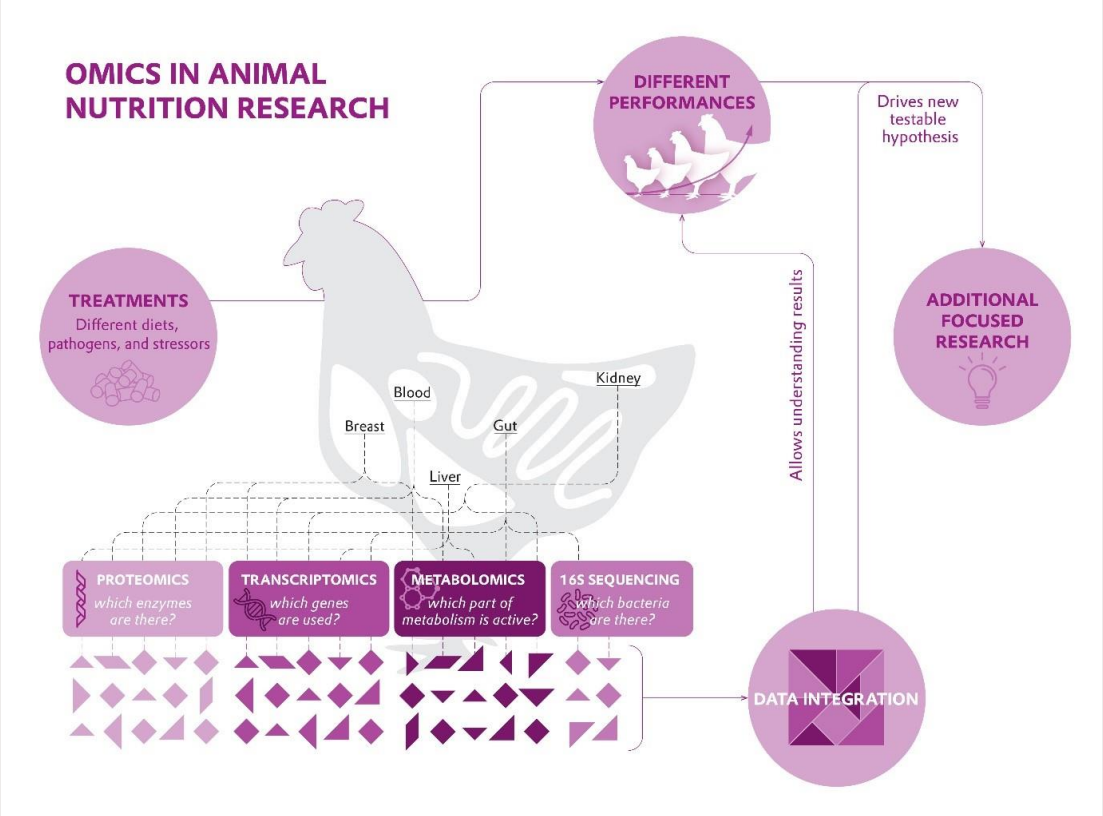

Figure 9: Omics methods in Animal Nutrition research. Treatments cause performance differences which are investigated with Omics methods. Taking type of treatment, performance differences and Omics results into account a new testable hypothesis for an underlying biological mechanism is formed. This leads to a new experiment focusing on testing that specific hypothesis.

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