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PROF. DR. KEZBAN ŞAHNA

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CONTENTS

CHAPTER 1

CONDITIONS IN GOATS AND SHEEP ASSOCIATED WITH SYMPTOMS OF THE RESPIRATORY SYSTEM

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INTRODUCTION

Sheep and goats are not only a solid source of income for people all over the world, but they are also an essential supply of protein for both human and carnivorous animal metabolisms. One of the key areas of research for experts around the world is how to use the products derived from these creatures with the least amount of harm (Hughes, 2001) Unfortunately, there is still no cure or vaccination for many of the viral infections that are regularly seen in sheep and goats, just like in other animal species. The most frequent viral illnesses of the respiratory system in sheep and goats include maedi-visna, enzootic penumonie, caprine herpes virus, ovine pulmonary adenomatosis, enzootic tumor, and caprine parainfluenza 3 (Batmaz, 2019). The information in the table below includes on the viruses that cause respiratory viral infections seen in sheep and goats, including their family, whether they are zoonotic or not, and whether vaccinations are available (Table 1). This review's goal is to describe the viral infections that affect sheep and goats, as well as the precautions that may be done to prevent them.

Table 1: *Name of the disease, family and genus of the agent, zoonotic status and availability of vaccines*

Name of viral diseases	Family	Genus	Zoonotic	Vaccine
Maedi-Visna	*Retroviridae*	*Lentivirus*	-	-
Enzootic penumonie	*Adenoviridae* *Reoviridae*	*Adenovirus* *Reovirus,* *Parainfluenza-3*	-	-
Caprine herpes virus	*Herpesviridae*	*Caprineherpesvirus*	-	+
Ovine pulmoner adenomatosis	*Retroviridae*	*Betaretrovirus*	-	-
Enzootik Tumor	*Retroviridae*	*Enzootic nasal tumor virus*	-	-
Caprine parainfluenza 3	*Paramyxoviridae*	*Respirovirus*	-	-

1. Maedi-Visna Virus

The OIE-World Organization for Animal Health classifies the Maedi-Visna virus (MVV) and the caprine arthritis encephalitis virus (CAEV) as small ruminant lentiviruses (SRLVs), under the Retroviridae family, which are notifiable animal illnesses. The progression and persistence of these infections have an impact on the welfare of animals as well as global trade (Benavides et al., 2015). The virus produces persistent degenerative alterations of smooth muscle hyperplasia (lungs), demyelination in the central nervous system, indurations of the udder, and proliferative synovial membrane modifications

(joints) during a protracted incubation period (Cecco et al., 2022). Clinical symptoms could include gradual weight loss, chronic respiratory conditions in sheep, or hard udders that produce less milk (Miguel et al., 2021).

Meadi-visna virus (MVV) primarily affects blood monocytes/ macrophages and dendritic cells, but it can also infect other cells, including epithelial cells and mammary glands, and it can spread from mother to child via free virus in colostrum (Borquez Cuevas et al., 2021). The virus limits its reproduction to blood monocytes, but when it enters the systemic circulation via a lymph node, it multiplies in mature tissue macrophage of the joint, mammary gland, and lung (Potarniche et al., 2020). When an animal contracts the MVV virus, it integrates into its leukocyte DNA and stays infected for the rest of its life. While the amount of the virus varies from animal to animal, both asymptomatic and sick animals can spread the MVV. Progressive interstitial pneumonia is used to identify Maedi, while meningoencephalitis is typically linked to Visna. Both names, which are Icelandic and translate to "dyspnea" (respiratory distress) and "wasting" (neurological symptom), respectively, come from the country's early 1940s research into both diseases. The only countries where Maedi has been recorded are Australia and New Zealand (De Andres et al., 2005).

Ovine progressive pneumonia (OPP) can be diagnosed in the field using clinical signs, macroscopic and microscopic lesions, and the detection of specific antiviral antibodies using the ELISA or AGID techniques; however, etiological detection is best accomplished using the classical or real-time version of PCR (Potarniche et al., 2020). The slow progression of OPP means that few infected sheep develop pathognomonic lesions; the most effective ways to identify OPP in herds are the detection of specific antibodies against the MVV in serum samples and conclusive diagnosis using molecular techniques (De Andres et al., 2005). The major threat to the widespread disease is not the existence of active surveillance programs but rather the lack of a national control program. Free trade of live small ruminants from various nations and locations where the disease has been reported may also be the main vector for its spread (Watt et al., 1992). A more organic experimental way of disseminating OPPV, aerosol nebulization may be useful for evaluating new vaccinations or particular host genetics. For the assessment of prospective vaccines and treatments, experimental infection models are crucial (Herrmann-Hoesing, 2010)

2. Enzootic Pneumonia of Lambs

Evidence from previous flu pandemics shows that secondary bacterial involvement frequently worsens primary viral lung infections and can progress to terminally impair lung function (Brown et al., 2014). Animals have also

shown this viral-bacterial synergy, particularly domestic ruminants, where stress and viral respiratory infections frequently contribute to subsequent bacterial pneumonia. It is taking some time to understand the precise processes that increase the pathogenicity of commonly commensal bacteria. Viruses may alter the function of alveolar macrophages or polymorphonuclear cells, decrease NK-cell activity, and/or increase the production of either pro- or anti-inflammatory cytokines in a way that is inappropriate for the clearance of the secondary bacterial infection (Shayakhmetov et al., 2005). These mechanisms may include virus-induced epithelial damage that exposes hidden bacterial binding sites.

The presence, type, and degree of any synergism that may exist between viruses and bacteria in the respiratory tract may have an impact on the safety of viral vectors in such a role, even though lung-directed viral gene therapy offers the potential to treat or ameliorate a variety of inflammatory, neoplastic, and inherited lung diseases. Although adenoviral vectors are frequently used in clinical trials, there is concern that their use could have unfavorable effects.

3. Caprine parainfluenza virus type 3

In nations where sheep are raised, respiratory infections are a significant economic and health concern. Economic losses are linked to not just fatalities but also convictions, slower growth, carcass degradation, and expenditures of prevention or treatment (Baghezza et al., 2021). Numerous animal viruses exhibit a highly distinct respiratory tropism. A singlestrand negative sense RNA virus with an envelope, caprine parainfluenza virus type 3 (CPIV3). They all belong to the Respirovirus genus of the Paramyxoviridae family, along with the human parainfluenza virus types 1 and 3 (HPIV1 and HPIV3, respectively), the bovine parainfluenza virus type 3, and the Sendai virus. According to reports, one of the primary viral respiratory infections in goats is CPIV3. Although this virus has the potential to cause pneumonia on its own, it more frequently contributes to the etiological complex of enzootic pneumonia (Kamdi et al., 2020). Figure 1 shows the clinical and pathological findings in a goat infected with the para influenza virus.

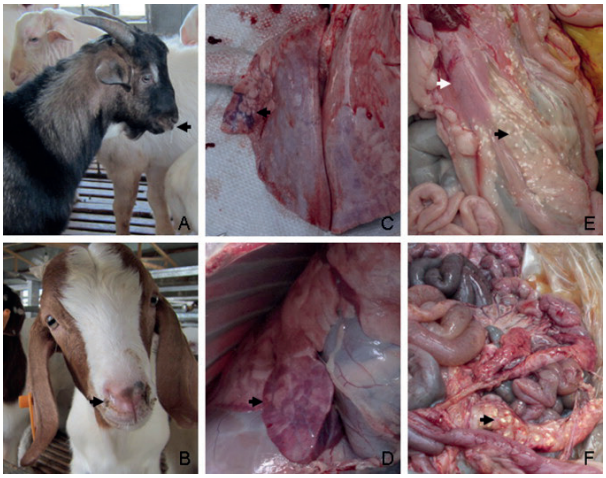

Figure 1. *Goats with disease: clinical and pathological observations. A and B are sick goats with nasal discharges; C and D are sick goats with mild to moderate widespread purple consolidation in their lungs; E is a sick goat with enlargement and bleeding in their pancreas; and E and F are sick goats with white punctiform tuberculum in their mesenterium (Li et al., 2014)*

Virus isolation in cell culture, polymerase chain reaction (PCR), and immunohistochemistry (IHC) investigations of the lower respiratory tract are used to make the definitive diagnosis of CPI3V infection (Kamdi et al., 2020). PIV3 is typically genotyped using several genomic areas, including the M, HN, and F genes. The M gene, which has been proposed as an appropriate target for genotyping, is the region that has been studied the most frequently. The virus was identified as CPIV3 in the Respirovirus genus and easily distinguished from the HPIV3 and BPIV3 strains using phylogenetic analysis. The full genome is now being sequenced in order to fully comprehend the genomic traits of this novel virus (Li et al., 2014). Serological techniques for identifying specific antibodies against PIV3 in goat and sheep herds should be developed, along with a comprehensive epidemiological examination of PIV3 in goats. Additional in-depth research is required to fully understand this novel isolate's pathogenicity, and relevant prevention strategies should be established.

4. Reovirus

The Reoviridae family of viruses, which presently has 15 identified genera within two subfamilies with genomes made up of several (10–12) segments of double-stranded RNA, is one of the most complicated in all of virology (Tan et al., 2017). A remarkable array of hosts, including mammals, birds, reptiles, amphibians, fish, mollusks, crustaceans, insects, plants, and fungi, are infected by specific viruses within the family. Bat-borne fusogenic

orthoreoviruses belonging to the Nelson Bay orthoreovirus species (family Reoviridae, subfamily Spinareovirinae, genus Orthoreovirus), commonly known as Pteropine orthereovirus (PRV), are also emerging reoviruses with compelling interest in human health. PRV produces acute respiratory illness or flu-like symptoms in humans (Di Teodoro et al., 2019). Although this infection has been characterized as being moderate and self-limiting, some patients have experienced severe disease, which can include a high fever, followed by a cough or sore throat, as well as some systemic symptoms such generalized weakness and myalgia. The Pteropodidae fruit bat, from which PRVs have predominantly been isolated (Di Teodoro et al., 2019).appears to be the direct source of human infection. Transmission from person to person has also been theorized (Tan et al., 2017). In South-East Asia, where fruit bat populations are high, PRV human infections are frequent..

5. Caprine Herpes-1 Virus

Herpesviruses are significant human and animal pathogens (Hao et al., 2020). Under a variety of circumstances, they have a serious negative impact on human health and cost many animal sectors money (Bertolini et al., 2018). Seven closely related viruses belonging to the Herpesviridae family's subfamily Alphaherpesvirinae and genus Varicellovirus are grouped together. They share similar antigenic characteristics, a wide range of hosts, including both domestic and wild ruminants, a short replication cycle, and the capacity to cause latent infection (Hao et al., 2020). One of the pathogens in this subfamily is caprine alpha-herpesvirus 1, or CpHV-1, which shares a genetic ancestor with bovine alpha-herpesvirus 1, or BoHV-1, which is known to cause infectious bovine rhinotracheitis (IBR), one of the major illnesses of cattle. Bovine alphaherpesvirus 1 (BoHV-1) and caprine herpesvirus 1 (CpHV-1) are related genetically and antigenically. Numerous serological studies have been carried out in sheep and goats from the past to the present to determine whether or not these species are capable of serving as a reservoir for BoHV-1, even though it is thought that BoHV-1 does not infect small ruminants (Hao et al., 2020). Studies are particular focused on goats, where both spontaneous and artificial BHV-1 infections have been observed. Because of the tight antigenic association between BoHV-1 and other alpha-herpesviruses and the potential for cross-reactions, serological test findings can occasionally be deceiving. This is especially true in nations where eradication campaigns are being carried out (Camero et al., 2017).

Although the infection's etiology is still not fully known, its symptoms were comparable to those of other members of the alpha subfamily of herpesviruses that caused genital lesions and latent infections (Bertolini et al., 2018). The virus is thought to infect goats by the respiratory or reproductive routes and has a high tropism towards the genital tract. Evidence shows that

CpHV-1 causes systemic disease in young kids with substantial morbidity and mortality, while it also causes respiratory disease, vulvovaginitis, and occasionally miscarriages in adult goats who are infected (Hao et al., 2020). For the diagnosis of CpHV-1 in serum samples, both the viral neutralization test (VNT) and the enzyme-linked immunosorbent assay (ELISA) can be used. Additionally, VNT is required to discriminate between BoHV-1 and CpHV1, although results are unreliable because both viruses react with one (Camero et al., 2017). CpHV-1 is managed on goat farms by prevention and eradication. Since the 2000s, various vaccination kinds have been researched. CpHV1 vaccinations have not, however, been made available due to the unprofitable nature of the pharmaceutical industry. As a result, hygienic preventive measures are required for the control of this infection, and alternate options require more research (Lilloet al., 2023). Mizoribine, an immunosuppressive medication, has been tested in vitro with aciclovir and shown to be effective against CpHV-1 [21]. Based on in vivo and in vitro experiments, the administration of cidofovir has also sparked attention for the treatment of genital lesions in the caprine species. The virucidal activity of ginger essential oil was discovered to inactivate CpHV-1 by up to 100% (Hao et al., 2020). A number of essential oils have also been examined for their ability to inhibit human viruses. However, there isn't much use of essential oils in veterinary medicine.

6. Ovine Pulmonary Adenomatosis

Ovine pulmonary adenomatosis (OPA), also called sheep pulmonary adenomatosis, is an infectious lung cancer in sheep that is brought on by the jaagsiekte sheep retrovirus (JSRV) (Shi et al., 2021). The virus causes neoplastic growth in alveolar cells (type II pneumocytes and club cells), which replaces healthy lung tissue, inhibits function, and increases fluid production (Crilly et al., 2022). JSRV is comparable to type B and type D retroviruses in morphology, biochemistry, and antigenicity, and it can survive in circulation for a number of years before infecting pneumocytes, where it induces neoplastic transformation (Shi et al., 2021). OPA affects many nations with a sheep breeding business economically, with the exception of Australia, New Zealand, the Falkland Islands, and Iceland (Zhang et al., 2014). Coughing, dyspnea, tachypnea, nasal discharge, loss of condition, and in rare circumstances, the generation of copious volumes of lung fluid are clinical indicators in OPA-infected animals with big tumors (Hofacre and Fan, 2010). A beta-retrovirus called exogenous JSRV (exJSRV) has a single-stranded, positive-sense RNA genome of around 7.5 kb and only the gag, pro, pol, and env essential genes that are typical of retroviruses (Zhang et al., 2014). The mature retroviral particle's structural layers are made up of the proteins capsid (CA), matrix (MA), and nucleocapsid (NC), which are encoded by

the group antigen gene (gag) (Hofacre and Fan, 2010). There is currently no effective treatment for OPA. Animals with OPA infection commonly develop secondary bacterial pneumonia, and sick animals typically pass within a few days to a few months following the commencement of the illness (Hofacre and Fan, 2010). OPA is one of the most significant illnesses affecting the worldwide animal trade, according to the Office worldwide des Epizooties (OIE). The development of the sheep industry in the local and surrounding areas is seriously threatened by the high incidence and prevalence of OPA in the Chinese provinces of Xinjiang and Inner Mongolia, where mortality rates are nearing 100% (Hsu et al., 2015).

Although sheep can contract JSRV at any age, the clinical indications of OPA are more frequently seen in two to four-year-old animals due to the lengthy incubation period (Grifths et al., 2010). Since the condition is usually only fully diagnosed after death, the clinical indications are generally not very specific. When OPA-affected sheep are examined postmortem, tumors typically show up as solid, grey-purple masses that largely damage the cranioventral lung areas (Hsu et al., 2015). Rarely, the tumor may seem dry, pale grey, or white. Additionally, the airways typically contain a significant amount of pulmonary epithelium lining fluid, which can be observed as a foamy discharge at the nostrils (Hofacre and Fan, 2010). On the other hand, few farmers submit their sheep for postmortem evaluation, and there is presently no commercially accessible test for use in live sheep, leading experts to believe that the disease is significantly underreported (Grifths et al., 2010).

JSRV cannot be propagated in vitro since there is no efficient cell culture system, which makes it more difficult to isolate and identify this virus (De las Heras et al., 2003). Since there is currently no serological test for JSRV, it is possible that the presence of enJSRV in the sheep genome causes host immunological tolerance. As a result, JSRV-infected animals do not exhibit any humoral immune response. But there are common assays for the clinical diagnosis of JSRV. A PCR assay has been proposed for the pre-clinical diagnosis of OPA in bronchoalveolar lavage fluid, but efficient and rapid detection methods for the clinical diagnosis of JSRV infection are still lacking. Additionally, it is challenging to identify JSRV-infected animals during the pre-clinical period (Grifths et al., 2010).

7. Enzootic Nasal Tumor

The infectious illnesses enzootic nasal adenocarcinoma (ENA) and ovine pulmonary adenocarcinoma (OPA) of goats and sheep are characterized by the transformation of ethmoid turbinate epithelial cells and the development of lethal lung tumors in young adult animals, respectively (Ye et al., 2019). OPA is brought on by infection with the ovine retrovirus Jaagsiekte sheep

retrovirus (JSRV), while ENA is thought to be brought on by The enzootic nasal tumor virus (ENTVs) 1 and 2, which have been successfully used in experiments to transmit enzootic intranasal tumors in sheep and goats. These viruses have been consistently found in nasal tumors and tumors (Ortín et al., 2003).

The enzootic nasal tumor virus is a single positive-stranded RNA virus that is a member of the family Retroviridae and the genus Betaretrovirus. While ENTV-2 is seen in goats, ENTV-1 is always found in sheep. JSRV and ENTV are both members of the family Retroviridae's genus Betaretrovirus. The ENTV genome is around 7.5 kb length and has a genomic architecture similar to type B and type D oncoviruses (De Cecco et al., 2019).

ENA naturally occurs everywhere, with the exception of Australia and New Zealand, and has been documented in numerous nations. Its incidence has even reached 10% in some locales (Allam et al., 2023) and in recent years, it has increased in China. Since 1995, when the first incidence of ENA in goats was discovered in Inner Mongolia, China, cases of ENA in goats have also been discovered in Hunan, Sichuan, Anhui, Shaanxi, Guizhou, and, most recently, Fujian. However, serological testing for the virus appears to be impossible due to the lack of circulating antibodies identified for ENA when employing antigens from natural sources, hence the prevalence and genotypic distribution of ENA are still unclear (Sid et al., 2018).

8. Conclusions

Strategy for creating safe vaccinations is to use non-replicating viruses as "viral-vectored vaccines," which contain the artificial gene that codes for the target protein. The virus vectors were enhanced on a genomic level to increase their capacity to carry many genes and their replication skills in order to tailor the required immune responses and induce long-lasting immunity. The worldwide COVID-19 pandemic significantly increased our understanding of this innovative class of vaccination. The focus is on the financial and technical factors, which further restricts their use in farm animal businesses despite the strong immune responses, lengthy history of in-lab successes, and wide range of uses in animal models.

8.1. Therapeutic Principles

Rapid identification of the underlying causes is essential for the diagnosis of any viral disease. The ideal diagnostic step is the isolation of the causing virus from the field samples. In the event that viral isolation is not possible, molecular science offers the possibility of more effective and trustworthy ways for diagnosing viral infection. The essential components of the control program include immunization against each virus, efficient quarantine

procedures, hygienic practices, and lastly vector control. When treating viral illnesses, the use of immunomodulators should be taken into consideration to improve non-specific defensive mechanisms. On the other hand, it's critical to track the effectiveness of viral disease treatments and look into any discrepancies. Other factors that need to be considered are the drug's negative effects and how long it stays in meat and milk. Treatment of the symptoms is crucial because the majority of viral diseases cannot be treated with antiviral medications (2, 3).

8.2. Conservation Principles

Reducing infection levels, removing risk factors for disease progression to stop epidemics, and boosting both specific and general immunity are all necessary. To mention this in brevity;

-Those who are affected should be isolated right once and should not re-join the herd until they have healed completely,

-Using the proper quarantine procedures,

-Colostrum should be given to newborns in sufficient amounts.

-Notifying notifiable diseases as soon as feasible - Testing new animals joining the herd for certain diseases by performing the relevant tests,

-The infection source must be found and eradicated; serological testing for the relevant disease in the herd must be carried out to determine the infection's stage,

-Shelters need to meet the necessary requirements,

-Instead of discarding the internal organs of deceased or autopsied animals into the environment, deep trenches should be dug and lime should be poured over them as needed,

-Animals should be kept as stress-free as possible,

-Viral diseases for which vaccines are available must be prevented through vaccination,

-Domestic carnivores and wild animals must be controlled, among other precautions

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CHAPTER 2

PRE-SYNCHRONIZATION PROTOCOLS IN DAIRY COWS

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INTRODUCTION

Dairy cows are aimed to produce one calf per year (Alan et al., 2000; Kalkan and Horoz, 2010; Xu et al., 2000). To achieve this goal, oestrus must be detected timely and accurately, cows should be inseminated at the correct time, and they must become pregnant as soon as possible (Kalkan and Horoz, 2010; Stephen et al., 1998). Recently, despite the use of the most modern techniques and technologies, the oestrus detection rate remains around 50% (Dinç and Kutlu, 2015). Failure to detect oestrus in farms practicing artificial insemination reduces insemination success and conception rates. Consequently, inadequate and inaccurate detection of oestrus is one of the major factors reducing reproductive fertility in cows (Lopez-Gatius and Vega-Prieto, 1990). Detecting oestrus during the postpartum period (PP) is particularly challenging (CEVA Turkey, 2014). More than 60% of the first oestrus cycles observed between 15 and 34 days postpartum are classified as suboestrus (CEVA Turkey, 2014). This condition is attributed to insufficient development of the corpus luteum (CL), leading to a shorter lifespan. Moreover, the short duration or silent (suboestrus) nature of oestrus in high-yielding cows complicates its detection. Another contributing factor is the presence of infertile cows (anoestrus, suboestrus, non-cyclic). Therefore, non-cyclic cows should be identified early during the second half of the postpartum voluntary waiting period (VWP). Furthermore, genital tract infections and metabolic problems delaying the onset of postpartum ovarian activity should be diagnosed early (Dinç, 2013; Hudson et al., 2012).

A useful criterion for assessing oestrus detection is the first oestrus observed after calving. The target should be 80% by the end of VWP (typically 60 days) (Dinç and Kutlu, 2015). The first observed oestrous after calving is a valuable criterion for evaluating oestrus detection. However, the oestrus detection rate alone may not be a fully accurate assessment tool. The best submission rate can be calculated using the oestrus detection efficiency of herds. Submission rate is the most common method used to determine oestrus detection efficiency. It is defined as the number of cows or heifers served within an 18- or 24-day period, expressed as a percentage of the number of cows or heifers that are at or beyond the earliest date at the start of the 18- or 24-day period (CAFRE, 2005; Dinç, 2013; Esslemont et al., 1985). The submission rate responds to changes in the herd much faster and demonstrates trends over time more effectively (Hudson et al., 2012). Cows that complete the VWP are included in the calculation of the submission rate (CAFRE, 2005; Dinç, 2013). The VWP determines the beginning of the submission rate. The target submission rate for farms with a year-round calving policy should be 75% per 3-week period (CAFRE, 2005; Dinç, 2013; Hudson et al., 2012).

Submission Rate = Number of cows served during specific period (18-24 days) / Number of eligible cows (18-24 days) × 100

Pre-Synchronization Protocols to Increase Submission Rate

Oestrus synchronizations may increase the submission rate (Nebel and Jobst, 1998). Ovulation synchronization protocols (Ovsynch), which do not require oestrus monitoring to increase the submission rate, can be applied to problematic cows whose oestrus cannot be detected or to farm with low oestrus detection rates. There are pre-synchronization methods that increase the effectiveness of these protocols. The aim of pre-synchronization is to initiate treatment early after calving, rather than waiting for cows to exhibit oestrus after the VWP and insemination. Using ovulation synchronization protocols, cows are inseminated without the need to observe their oestrus. When this treatment is applied, the oestrus cycle of the cows will be regular and they show oestrus every 18-24 days, even if they do not become pregnant after the first insemination. Therefore, there will be at least 3 or 4 chances for insemination by the 125th day after calving. Different methods are used to increase the submission rate. Information about hormonal applications developed to enhance the submission rate is presented below. In this context, hormonal applications such as Presynch, Double-Ovsynch, G6G, PG-3-G, and PG+G are significant (Figure 1 and Table 1).

If ovulation synchronization protocols are initiated during the early luteal phase of the oestrus cycle (in the presence of a dominant follicle—dioestrus between days 5 to 9 of the oestrus cycle), a high pregnancy rate can be achieved. The best response is observed when a dominant follicle with a diameter of 10 mm is present. Cows that began Ovsynch on days 5 to 9 also exhibited greater circulating P4 levels at the time of PGF2α treatment (3.6 ng/ml), likely due to the presence of two CL, compared to cows that initiated Ovsynch on days 1 to 4 (2.5 ng/ml) (Moreira et al., 2000; Souza et al., 2008; Wiltbank and Pursley, 2014). In cows that began Ovsynch on days 1 to 4, the first GnRH treatment rarely resulted in ovulation. Consequently, cows on the day 1 to 4 schedule developed an older and larger follicle that ovulated in response to the second GnRH treatment (19.2 mm), compared to cows that began Ovsynch on days 5 to 9 (16.8 mm). When the Ovsynch protocol is initiated on days 15 to 21 of the oestrus cycle, cows may be in oestrus during the PGF2α injection. In this case, the follicle is small and does not develop a new CL, resulting in a low P4 concentration (Vasconcelos et al., 1999; Wiltbank and Pursley, 2014).

Pre-synchronization methods (Presynch) containing only PGF2α may fail to stimulate oestrus and increase fertility in anovular, non-cyclic animals (Bisinotto et al., 2014). This is a common issue in high-yielding cows after the VWP, with rates ranging from 7.1% to 41.7% (Bamber et al., 2009). Since PGF2α cannot alter the course of follicular development, differences in oestrus and ovulation timing caused by the developmental phase of the follicular wave may lead to complications when applied. To address this, pre-synchronization protocols that combine PGF2α and GnRH, such as Double-Ovsynch, PG +

G, G6G, and PG-3-G, have been developed. These protocols synchronize the onset of the Ovsynch protocol with the early luteal phase of the oestrus cycle (in the presence of a dominant follicle—dioestrus between days 5 to 9 of the oestrus cycle), resulting in a high pregnancy rate in anovular cows.

Presynch

The Presynch protocol positively affects fertility by increasing the number of oestrus, improving the uterine environment and immune system, and enhancing embryo survival (Cavalieri et al., 2006). It was first applied by Moreira et al. (2001) (Moreira et al., 2001). The aim of Presynch is to achieve a 100% submission rate by the end of the VWP and ensure that animals are inseminated as soon as possible. Before starting the ovulation synchronization protocol, PGF2α is administered twice at 14-day intervals (Figure 1). In the original Presynch protocol, ovulation synchronization methods are initiated 12 days after the second PGF2α administration (Moreira et al., 2001). If cows respond to the first PGF2α, they enter the luteal stage and respond to the second PGF2α 14 days later. If cows do not respond to the first PGF2α, they will enter the luteal stage within 14 days and then respond to the second PGF2α. During Ovsynch, which begins 14 days after the second PGF2α administration, cows are typically between days 5–14 of the oestrus cycle. When Presynch-14, Presynch-12, Presynch-11, or Presynch-10 is used as a pre-synchronization program, cows are generally between days 9–14, 7–12, 6–11, and 5–10 of the oestrus cycle, respectively, at the time ovulation synchronization begins (Stevenson et al., 2012). Presynch is a pre-synchronization method commonly used during the PP)period. Early initiation of PP administration may reduce problems such as ovarian cysts and pyometra, while increasing luteal activity, oestrus, ovulation, and pregnancy rates (Lopez-Gatius and Vega-Prieto, 1990). Presynch can stimulate oestrus in up to 75% of anovular animals (Thatcher et al., 2004). Presynch-Ovsynch should be regarded as a comprehensive treatment method rather than merely a tool to increase pregnancy rates in high-yielding cows (Wiltbank and Pursley, 2014).

Double-Ovsynch

In the Ovsynch protocol, cows are not found to be in appropriate stages of the cycle in terms of follicular development and lower success is achieved in non-cyclic cows. To address these limitations, the Double-Ovsynch protocol has been developed, which partially overcomes these disadvantages (Souza et al., 2008). The initiation of the second Ovsynch protocol in the Double-Ovsynch protocol during days 5–12 of the oestrus cycle significantly improves synchronization rates. Compared to cows treated with the Ovsynch protocol alone, the Double-Ovsynch protocol offers the advantage of eliminating the need to determine the stage of the oestrus cycle at the start of the protocol (Bilgen and Özenç, 2010). The Double-Ovsynch protocol posi-

tively affects fertility by increasing the number of cows in the early dioestrus stage at the start of the second Ovsynch administration and by raising serum progesterone concentrations during the protocol (Souza et al., 2008). In Double-Ovsynch protocol, ovulation occurs after the first administration of GnRH, followed by the formation of a corpus luteum (CL) and the emergence of a new follicular wave. This is similar to the process observed after the first GnRH injection in the Ovsynch protocol. However, the Double-Ovsynch protocol offers additional benefits, including ovulation before timed artificial insemination (TAI). Seven days after the first administration of PGF2α, the CL regresses, and ovulation occurs. Three days later, a second administration of GnRH induces a new follicular wave. Seven days after that, a third GnRH injection induces ovulation again. At this point, there are likely two CLs in the ovaries: one formed by the second GnRH and the other by the third GnRH. Seven days later, the administration of PGF2α causes the regression of these two CLs. A fourth GnRH injection is then administered to stimulate ovulation, followed by TAI. The primary advantage of this protocol is that it is 11 days shorter than the classic Presynch-14 protocol. The high progesterone concentration in the follicular environment in response to the third GnRH administration leads to more cows ovulating and being fertilized after TAI. Both factors—enhanced ovulation rates and progesterone enrichment in the follicular environment—have been shown to improve fertility outcomes with TAI. Additionally, about 40–45% of cows show oestrus during TAI, and the protocol also facilitates the initiation of the first ovulation in anovular cows through the administration of either the first or second GnRH before starting Ovsynch. However, the primary disadvantage of the Double-Ovsynch protocol is its higher cost compared to the Presynch-Ovsynch protocol (Astis and Fargas, 2013; Carvalho et al., 2014; Herlihy et al., 2012; Stevenson, 2012b).

G6G

In this protocol, the goal is to regress a young, middle or old CL that may be present in ovaries by starting with a PGF2α injection. Forty-eight hours after the injection, GnRH is administered to induce ovulation and the formation of a new CL. The purpose of these two consecutive applications is to initiate a new synchronized oestrus cycle and stimulate a new wave of follicular growth. This pre-synchronization program ensures the presence of a functional dominant follicle ready for ovulation, which will respond to the first GnRH injection of the Ovsynch protocol, initiated six days later. The new CL formed by the G6G protocol increases progesterone concentrations during the seven-day follicular development and maturation period leading up to TAI. These elevated progesterone levels positively affect follicle development and oocyte quality (Astis and Fargas, 2013; Bello et al., 2006; Stevenson, 2012a; Yılmaz et al., 2011). When the G6G protocol is applied, the Ovsynch protocol begins during days 4, 5, or 6 of the oestrus cycle (Pursley, 2015; Ste-

venson, 2011). The G6G protocol is particularly suitable for cows that do not have a CL present during examination (Pursley and Martins, 2011), multiparous cows undergoing their first insemination (Astis and Fargas, 2013) and anovular cows (Ribeiro et al., 2011).

PG-3-G

Cows treated with the PG-3-G protocol were injected with PGF2α 10 days and GnRH 7 days before the start of the Ovsynch protocol. In this protocol, 90% of cows are in the middle of the luteal phase at the beginning of the ovulation synchronization protocol. Therefore, cows with a dominant follicle in the early stages of a follicular wave (<10 mm) at the time of PGF2α administration would likely develop a follicle with an antral diameter of ≥10 mm and have LH receptors at the time of the subsequent GnRH injection 3 days later, responding with ovulation (Peters and Pursley, 2002). When the G6G protocol is applied, the Ovsynch protocol begins during days 5–6 of the oestrus cycle (Stevenson, 2011). The PG-3-G protocol increases the ovulation rate and 7-day luteal function before Ovsynch, resulting in better synchronization of follicular growth and improving the potential to increase pregnancy rates (Stevenson et al., 2012).

PG+G

The PG + G protocol involves the simultaneous administration of GnRH and PGF2α injections 7 days before the start of Ovsynch. Pregnancy rates are similar when compared to the G6G protocol; however, the potential progesterone concentration during ovulatory follicle growth is lower. In this protocol, the simultaneous injections of GnRH and PGF2α at the beginning reduce labor costs and shorten the duration of the protocol compared to the G6G and PG-3-G protocols. It serves as an alternative to more complex pre-synchronization protocols (Martins et al., 2017; Yousuf et al., 2016).

Result

As a result, the choice and effectiveness of the pre-synchronization program to be used will depend on the farm's resources, objectives, and the veterinarian's preference (Wiltbank and Pursley, 2014). It is important to note that GnRH administration can potentially reduce oestrus in cows when pre-synchronization programs are implemented (Chebel et al., 2013; Lopes et al., 2013; Mendonca et al., 2012). On the other hand, the duration of the pre-synchronization period before TAI should not pose a significant issue. The main considerations are the costs, the level of fertility on the farm, and the effectiveness of farm management.

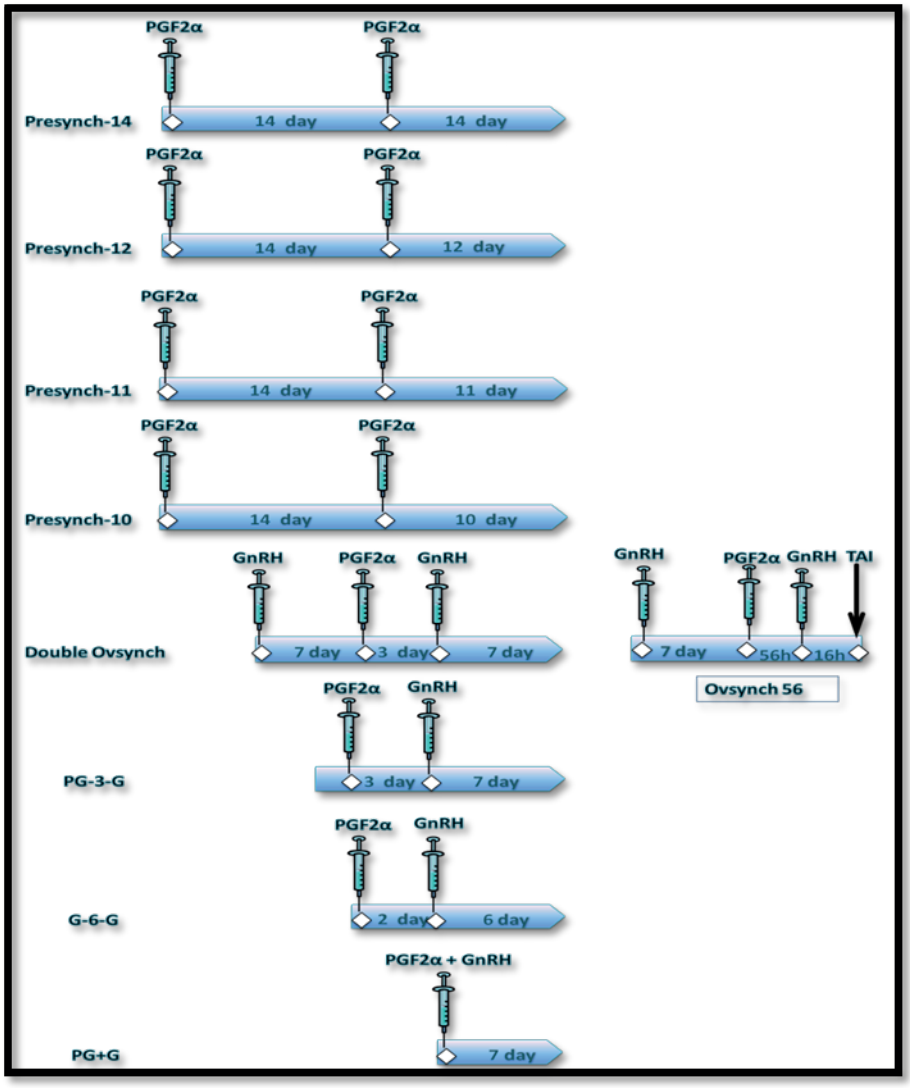

Figure 1. Pre-synchronization protocols

Table 1. *Pre-synchronization protocols pregnancy rates*

Gebe Kalma Oranları	Ovsynch	Presynch	Double-Ovsynch	G6G	PG-3-G	PG+G
Moreira et al. (2001) (PP 37)(All Cows)	6% (n=97)	36.9% (n=88) (Presynch-12)				
Moreira et al. (2001) (PP 37) (Cycle Cows)	34.4% (n=67)	**46.9%** (n=66) (Presynch-12)				
El-Zarkouny et al. (2004) (PP 59-79)	37.5% (n=304)	**46.8%** (n=310) (Presynch-12)				
Navanukraw et al. (2004) (PP 60>)	37.3% (n=134) (PP 99)	**49.6%** (n=135) (PP 89) (Presynch-14)				
Aköz et al. (2008) (n=104) (PP 26-41)		43% (n=54)(Presynch-14)				
Carvalho et al. (2014) (n=661)			**52.6%** (n=306)			
Herlihy et al. (2012) (n=1687) (PP 57-68)		Primipar=42.3% Multipar=34.3% (Presynch-12)	Primipar=**52.5%** Multipar=40.3%			
Souza et al. (2008) (PP 42>)		Primipar=45% Multipar=39% (Presynch-12) (n=180) (PP 42±3)	Primipar=**65.2%** Multipar=37.5% (n=157) (PP 51±3)			
Bilgen and Özenç (2010) (n=51) (PP 25, 32, 39)			Primipar=**75% (PP 32)** Primipar=25%(PP 25,39) Multipar=**75%(PP 39)** Multipar=37.5%(PP 25)			
Astiz and Fargas (2013) (n=7805) (PP 87±10)			Primipar=**44.3%** Multipar=31.4% (n=6783)	Primipar=34.7% Multipar=34.8% (n=1022)		
Bello et al. (Bello et al.) (PP 62-72)	27% (n=34)	-	-	**50%** (n=32)		

Yılmaz et al. (2011) (Cows) (PP 50-100)	37.8% (n=37) (PP 83±2)	-	-	**53.8%** (n=119) (PP 88±3)	
Pursley and Martins (2011)				61.4% (n=800)	
Riberio et al. (2012)				28.7% (n=178) 5-day cosynch72 (PG) **45.4%** (n=185) 5-day cosynch72 PG$^{1/2+1/2}$)	
Riberio et al. (2011)		49.1% (n=632) (Presynch-11)		**49.9%** (n=625) 5-day cosynch72 (PG$^{1/2+1/2}$)	
Sonat et al. (2014) (PP 45-90)				45% (n=40)	
Dirandeh et al. (2015)				39.5% (n=250)	
Heidari et al. (2017) (PP 28 ± 3)				32.9% (n=149)(PG) 37.5% (n=144) (PG$^{1/2+1/2}$)	
Peters and Pursley (2002) (PP 49-55) (n=427)	%38.3				**41.5%**
Stevenson et al. (2012)		33.3% (n=105) (45.7% cold season) (n=71) (PP 50) (Presynch-11)			**40%** (n=105) (59.1% cold season) (n=66) (PP 36)
Stevenson and Pulley (2012) (PP 42)		35.0% (n=1247) (44.3% cold season) (26.7% hot season) (Presynch-10)			**41.2%** (n=1286) (46.8% cold season) (n=465) (35.9% hot season) (n=421)
Pulley et al. (2015)		52.6% (n=19) (GnRH 56) 22.2% (n=18) (GnRH 72) (Presynch-10) (PP 34)			**57.1**% (n=14) (GnRH 56) 56.3% (n=16) (GnRH 72) (PP 45)

Stevenson et al. (2018)		39.1% 7-day ovsynch (PG) 30.0% 7-day ovsynch (PG1/2+1/2) 35.0% 5-day ovsynch (PG) 38.7% 5-day ovsynch (PG1/2+1/2) (n=194)		**52.1**% 7-day ovsynch (PG) 44.0% 7-day ovsynch (PG1/2+1/2) 25.2% 5-day ovsynch (PG) 39.4% 5-day ovsynch (PG1/2+1/2) (n=201)	
Kutlu (2015) (PP 28-40)			42.9% (n=35)	**51.4%** (n=35)	
Yousuf et al. (2016) (PP 58-64)			**57%** (n=114)		50% (n=121)
Martins et al. (2017) (PP 41-47)	**%48** (n=214) (Presynch-10)				%46 (n=218)

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CHAPTER 3

PREBIOTIC OLIGOSACCHARIDES AS FEED ADDITIVES IN FISH FARMING

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Introduction

In addition to other livestock and products obtained from these animals, fish farming is a rapidly growing sector of particular importance in meeting the animal protein needs of the world population. In fact, the aquaculture industry provides about half of all aquaculture products consumed by the public (FAO, 2021). As in every production area, in the aquaculture industry, while attempts are made to achieve maximum profitability, it is aimed to obtain safe products in terms of both environment and public health (Yilmaz *et al*., 2022). However, factors such as dense housing, poor water quality and human-induced stress not only create a breeding ground for the spread of infections in animals, but also negatively affect the growth performance and immune system of animals, making them vulnerable to infections that cause economic losses (Tort, 2011; Pérez-Sánchez *et al*., 2018).

The addition of antibiotics to feed as growth promoters and for control of diseases in animal production for many years has led to discussions of the risks to public health and legal challenges, and for this reason the use of all sub-therapeutic antibiotics as growth-enhancer in feed was banned in the European Union in 2006 (Denev *et al*., 2009). Public health risks also exist for aquaculture, and potential problems caused by uncontrolled antibiotic use include the development of bacteria resistant to antimicrobials, the creation of resistant genes, antibiotic residues in aquaculture products and their unsuitability for human consumption, environmental residues, and a decrease in beneficial microbiota in the gastrointestinal tract (Butt *et al*., 2021; Munir *et al*., 2016).

As a result of concerns about the use of antibiotics as feed additives and the legal restrictions and prohibitions, studies on alternative strategies to overcome this deficit have accelerated and research in this direction is still continuing today. In this context, in addition to livestock production, in the field of aquaculture, interest in alternatives such as prebiotics, probiotics, synbiotics and parabiotics continues today, as well as many natural feed additives that do not have negative effects on animal and consumer health. Although many papers have been published on the use of probiotics in aquaculture, their large-scale use has been limited due to their high cost, potential environmental impacts and regulatory issues, and as an alternative, manipulation of the gastrointestinal tract microbiota with prebiotics has attracted attention as a more practical approach (Ringø *et al*., 2010). In this chapter, it is aimed to provide general information on the use of oligosaccharides as prebiotics in fish farming applications and to mention the results of studies on this subject.

Mechanism of Action of Prebiotics

Prebiotics were first defined in 1995 as nondigestible food ingredients that selectively stimulate the growth and activity of a limited number of

bacterial species present in the host's intestinal microflora, thereby promoting improvement in the host's health (Gibson and Roberfroid, 1995). Prebiotics can also be used as an energy source by intestinal bacteria and are also known as functional saccharides (Song *et al*., 2014). The use of prebiotics as an energy source by intestinal flora leads to a decrease in intestinal pH due to the formation of short-chain fatty acids such as lactic acid, acetic acid, propionic acid and butyric acid in the environment, an increase in mineral absorption (especially calcium) and also the use of the compounds released as a result of fermentation as an energy source by intestinal epithelial cells (Özden, 2010). Prebiotics are carbohydrates classified according to their molecular size and degree of polymerization and the degree of polymerization refers to the number of monosaccharide units (monosaccharides, polysaccharides, oligosaccharides) (Akhter *et al*., 2015).

While all prebiotics are fibers, not all dietary fibers have prebiotic properties and some dietary fibers exhibit prebiotic effects by selectively stimulating the growth and activity of intestinal bacteria with potential for health. The effects of prebiotics on health include preventing damage from pathogens in the gastrointestinal tract, improving intestinal barrier function, reducing the population of pathogenic bacteria, production of beneficial short-chain fatty acids that modulate the immune system, production of pro-inflammatory cytokines, positive effects on mineral bioavailability, reduction in blood lipid levels and effects on insulin resistance (Guarino *et al*., 2020).

In order for a nutrient component to be accepted as a prebiotic, it should I) be hydrolyzed and absorbed in the upper part of the intestinal tract, II) be used by beneficial bacteria in the intestine, III) be able to modify the intestinal flora in a health-promoting way, IV) have a positive effect on health (Bakır, 2012). In addition, prebiotics (a) should be easy to incorporate into feed or ration, (b) should regulate intestinal viscosity, (c) should not be carcinogenic, (d) should be derived from dietary polysaccharides, (e) should have low calorific value. (f) reduce harmful microbial loads, (g) be effective at low concentrations, (h) exhibit anti-adhesive properties against harmful gut microbes, (i) stimulate beneficial gut microbes, and (j) have no lasting effects (Ganguly *et al*., 2010).

When using prebiotics in fish diets, it should be kept in mind that the effect of each prebiotic on fish may be different, and the effects of prebiotics may vary depending on various factors such as the structure of the prebiotic, dose, period of addition to the diet, species, age, period, weight of the fish. In addition, it should be taken into consideration that the addition of prebiotics to the diet should not be done indiscriminately and the limits should be paid attention, and that the use of prebiotics in the medium or long term may have adverse effects on the growth and health of the fish, as well as increase the cost of production (Zhu *et al*., 2023).

Fructooligosaccharides (FOS)

FOS, which are simple carbohydrates, are also known as Inulin-type oligosaccharides and have high solubility in water and high stability between pH 4.0-7.0 (Molina *et al*., 2009). Inulin is currently obtained from the roots of the chicory plant, and it is estimated that approximately 36,000 plant species contain inulin (Flickinger *et al*., 2003). In addition to chicory, which contains 15-20% inulin in its roots, it has been reported that inulin ratios are between 2-6%, 14-19%, 3-10%, 9-16%, 3-10% and 0.3-0.7% in the tuber parts of plants such as onion, Jerusalem artichoke, leek, garlic, artichoke leaves and stems and banana fruit, respectively, and between 0.5-1% and 0.5-1.5% in rye and barley, respectively (Franck & De Leenheer, 2005). Therefore, the same natural resources are also considered to be rich in FOS. Although FOSs can be obtained from inulin-rich plants by extraction, enzymatic degradation of inulin or enzymatic synthesis from sucrose, the majority of commercially available FOSs are synthesized from sucrose by the action of fructosyltranferases or by enzymatic degradation of inulin (Rastall, 2010; Bali *et al*., 2015; Ganaie *et al*., 2014; Singh & Singh, 2010; Mutanda *et al*., 2014). Basically, FOS obtained from plants is made up of terminal fructose attached to a glucose residue through β-(1→2) glycosidic bonds. It comprises 3-10 fructose units and has a non-reducing sucrose end. FOS and inulin are sorted according to their degree of polymerization (DP), which varies with the number of monosaccharide units. FOS has a DP below 10, whereas inulin's DP spans from 2 to 60. (Kumar *et al*., 2018).

As much as there have been studies on the use of FOS as prebiotic oligosaccharides in fish farming in the past years, the interest in this prebiotic still continues today. In almost all studies evaluating the effects of FOS on various fish species, parameters such as growth performance, survival rate, stress and disease resistance, immune system, metabolism, antioxidation and gut microbiota are common parameters. Although the results obtained from the studies are variable, it is noteworthy that recent studies have proven the positive effects of FOS in fish. In rainbow trout (*Oncorhynchus mykiss*), as a result of feeding with diets in which inulin and FOS were added at a rate of 0.5 and 1% each for 49 days, body weight gain in the experimental groups was significantly higher than the control, but there was no significant difference between the experimental and control groups in terms of feed intake and feed conversion rate (Ortiz *et al*., 2013). In the same experiment, intestinal Ca absorbtion was significantly higher in the groups supplemented with these prebiotics than the control group. FOS supplementation at a level of 1.0, 2.0 and 4.0% to per kg diets of tilapia (*Oreochromis niloticus* × *O. aureus*) for 8 weeks, final weight, percent body weight gain, specific growth rate, feed conversion ratio, the hepatosomatic index and viscerosomatic index in terms of performance parameters were significantly improved compared to the control

group. As serum immunological parameters, serum alkaline phosphatase level was significantly increased in all FOS groups except serum nitric oxide levels, and serum lysozyme level was significantly increased only in the group given 1.0 g/kg dietary FOS compared to the control group (Poolsawat *et al*., 2020). In this study, researchers also stated that the FOS supplementation significantly increased hepatic superoxide dismutase activity, amylase activity and villus height in the anterior part of the intestine, and reduced mortality against *Aermonas hydrophila* infection. As a result, they also reported that adding at least 1% FOS to the diet in tilapia could increase growth performance, nutrient utilization, immunological and antioxidant status, digestive enzyme activity, intestinal health and disease resistance. In another 56-day study conducted by Panase *et al*. (2023) on the same fish species, the addition of 1, 3 and 5 g/kg FOS to the diet did not cause a significant difference in terms of performance parameters and survival rate, but in terms of immune parameters, lysozyme activity was significantly higher in the 5% FOS group and respiratory burst activity was significantly higher in the groups supplemented with 1% and 3% FOS than the control group. In addition, it was determined by the immune-related gene expression tests that the relative gene expression of liver complement C3, interleukin 1 beta (IL-1β), tumor necrosis factor (TNF-α), interferon gamma (IFN-γ) and heat shock protein 70 (hsp70) was statistically highest in the 5% FOS group among the three FOS levels. In an 8-week experiment conducted by Yuan *et al*. (2022) on common carp (*Cyprinus carpio*), the group supplemented with 0.3% FOS had significantly higher final weight, percent of weight gain, specific growth rate and significantly better feed conversion ratio than the control group. In terms of blood immune index, except for complement 4 content, no significant difference was found in plasma complement 3 content, lysozyme, acid phosphatase, alkaline phosphatase activities, and in terms of antioxidant index, no significant difference was found in liver superoxide dismutase, catalase, glutathione peroxidase, malondialdehyde activities and total antioxidant capacity compared to the control group, but in terms of protease, lipase and amylase, 0.3% FOS group had significantly higher enzyme activities than the control group.

There is also evidence that dietary FOS supplementation has a stress-relieving effect in fish under stress. Soleimani *et al*. (2012) observed that the addition of 1, 2 and 3% FOS to the diet in water with a salinity level of 150 g/L significantly increased the survival rate of Caspian roach (*Rutilus rutilus*). In an experiment conducted by Zhang *et al*. (2021) to investigate the effects of FOS against oxidative stress and immune suppression induced by triphenyltin in goldfish (*Carassius auratus*), 0.4% and 0. 8% FOS supplementation significantly ameliorated triphenyltin-induced immune toxicity, and it was successful in reducing oxidative stress and immune suppression by partially preventing changes in antioxidant enzyme activities and expression of antioxidant and

ROS scavenger-related genes, and by significantly suppressing the production and mRNA expression of TNF-α, IL-6, and IL-1β. Addition of 1% and 2% FOS to *Labeo rohita* diets under heat stress (40°C) significantly increased final weight, body weight gain and survival rate without any change in daily feed intake in terms of performance, and protease, amylase, lipase levels in terms of digestive enzymes compared to the control group, and as a result, it was reported that the addition of FOS to the diet had a reducing effect on the negative effects of heat stress in fish (Gulzar *et al*., 2024).

Results have also been reported that adding FOS to fish diets has positive effects on the intestinal microbiota and increases the beneficial bacterial population. It was thought that the increase in the amount of lactic acid bacteria, which are considered as beneficial bacteria in the intestine, could possibly be a result of the role of FOS in the supply of enzymes, RNA and free nucleotides, B-complex vitamins and amino acids that are effective in the development of these bacteria (Hosseinifar *et al*., 2011). In common carp (*Cyprinus carpio*) supplemented with 1, 2 and 3% dietary FOS for 7 weeks, the total culturable autochthonous bacteria level increased significantly, whereas only 2 and 3% FOS supplementation was effective in increasing the number of autochthonous lactic acid bacteria significantly compared to the control group (Hoseinifar *et al*., 2014). On the other hand, in the same fish species, the addition of 0.5% and 1% short-chain fructooligosaccharides (scFOS) to the diet for 7 weeks did not cause a significant difference in the level of total culturable autochthonous bacteria, but significantly increased the level of autochthonous lactic acid bacteria (Hoseinifar *et al*., 2016). Addition of 1% and 2% FOS to stellate sturgeon (*Acipenser stellatus*) diets for 11 weeks significantly increased the total culturable autochthonous bacteria level, but only 1% FOS level was effective in significantly increasing the lactic acid bacteria level (Akrami *et al*., 2013). A recent experiment on tilapia (*Oreochromis niloticus* × *O. aureus*) is noteworthy in showing that FOS can produce similar effects at lower levels. In this experiment, FOS was added to tilapia diets at 0.05, 0.1, 0.2 and 0.4% levels for 8 weeks and in these experimental groups, total bacterial count increased significantly only in 0.2 and 0.4% FOS group, lactic acid bacteria count increased significantly only in 0.4% FOS group and *Bacillus* count was significantly higher in all FOS groups compared to the control group (Poolsawat *et al*., 2020).

Galactooligosaccharides (GOS)

Galactose, which contains oligosaccharide in its structure, is naturally present in milk and GOS, which has prebiotic effect, is divided into two groups as α and β-GOS due to differences in galactosidic bonds (Tian *et al*., 2019). Today, the most widely produced and used in research is β-GOS, which is synthesized by lactose transgalactosylation reaction using the enzyme β-galactosidase (Mei *et al*., 2022). Commercially available GOS products

are a mixture of galactose-based oligosaccharides with varying degrees of polymerization and linkage configuration with glucose, galactose and lactose (Torres *et al*., 2010). GOS produced by enzymatic transglycosylation using β-galactosidases or β-glucosidases enzymes represents β (1-4) GOS in almost all scientific studies and is sometimes used as trans-galactooligosaccharide (TOS) as an abbreviation (Bruno-Barcena & Azcarate-Peril, 2015). GOS, one of the natural oligosaccharides, has been reported to improve the intestinal barrier as a prebiotic, reduce the colonization of pathogenic bacteria in the intestine, regulate the intestinal microbiota by increasing the colonization of beneficial bacteria such as Bifidobacterium and Lactobacillus, improve lipid metabolism and prevent bone loss by increasing mineral absorption (Tian, 2019).

In aquaculture, the application of GOS has increased considerably due to their effects on fish growth performance, antioxidant capacity, immunity, intestinal microbiota and disease resistance (Xu *et al*., 2022). In a 6-week experiment carried out by Miandare *et al*. (2016) on goldfish (*Carassius auratus gibelio*), 0.5, 1 and 2% GOS were added to the diet, and at the end of the experiment, it was determined that GOS supplementation had no significant effect on growth performance, however, in terms of serum innate immune parameters, GOS supplementation significantly increased total protein, albumin, globulin, lysozyme and alkaline phosphatase activity, especially 2% GOS supplementation was more effective than other groups in terms of skin mucus immune response. In addition, it has also been reported that addition of GOS to the diet increases the gene expression of inflammatory cytokines TNF-1α and TNF-2α, but decreases appetite-related ghrelin gene expression. In a study performed by Buentello *et al*. (2010) on juvenile red drum (*Sciaenops ocellatus*), 1% trans-galactooligosaccharide (TOS) was added to the diet and exposed to *Amyloodinium ocellatum* after a 4-week feeding period to evaluate the non-specific immunity after 2 weeks. There was no significant difference between the TOS supplemented group and control in terms of weight gain and feed efficiency, but survival rate was 78% in the TOS supplemented group and 58% in control. In terms of non-specific immune response, serum lysozyme and intracellular superoxide anion production were significantly higher in the TOS group compared to control. In another experiment with the same fish species, the addition of 1% TOS to the diet was evaluated in terms of digestive enzymes and gastrointestinal canal histological parameters, and after 8 weeks, there was nonsignificant difference in digestive enzymes (pepsin, trypsin, chymotrypsin, aminopeptidase, α-amylase, lipase, alkaline phosphatase, acid phosphatase) levels, but anterior intestinal fold length, enterocyte height and microvillus height were significantly higher than control (Anguiano *et al*, 2013). Recently, in a 92-day trial on African catfish (Clarias gariepinus) (Genç *et al*, 2020), it was reported that the addition of two

different levels of GOS (0.1% and 0.2%) to the diet produced significantly better results in terms of final weight and final total length, only 0.2% GOS group was significantly higher than the control group in terms of weight gain and feed conversion ratio, and only 0.1% GOS group was significantly higher than the control group in terms of daily weight gain and survival rate, while there was no significant difference in terms of specific growth rate, hepatosomatic index and viscerosomatic index. In an 8-week study carried out by Xu *et al.* (2022) to investigate the effects of GOS supplementation at 1, 3 and 5% levels in juvenile hybrid sturgeon (Acipenser baerii♀ × A. schrenckii♂), final body weight, body weight gain, specific growth rate, feed intake and feed conversion ratio were significantly better than the control group, but nonsignificant differences were found for hepatosomatic index and viscerosomatic index. In the same study, serum immune related enzyme activities (acid phosphatase, alkaline phosphatase, lysozyme, myeloperoxidase) were significantly higher in the experimental groups than control. In terms of liver antioxidant responses, only glutathione peroxidase was significantly higher in the experimental groups than control, while superoxide dismutase and catalase activities were significantly higher only in the 3 and 5% GOS groups than the other groups. Although malondialdehyde levels were significantly lower in the experimental groups compared to the control, the lowest malondialdehyde level was detected in the 5% GOS group. In a 6-week trial on Caspian roach (Rutilus caspicus) and Caspian white (Rutilus kutum) fingerlings to investigate the effect of GOS as a prebiotic on intestinal microbiota (Hoseinifar *et al*, 2019), the addition of 1% and 2% GOS to the diet did not cause a significant difference in total bacterial counts, but significantly increased lactic acid bacteria levels and significantly increased the ratio of lactic acid bacteria to total viable bacteria in the intestinal microbiota.

In recent years, there have also been studies on common carp that provide data on GOS. In a 60-day experiment conducted by Hoseinifar *et al.* (2017) in common carp (*Cyprinus carpio*), final weight, weight gain, specific growth rate and feed conversion ratio values significantly improved in the groups fed diet containing 2% GOS compared to the control group. In addition, in the same study, GOS group had significantly higher blood respiratory burst activity, mucus total Ig level and lysozyme activity, serum lysozyme alternative complement activity, serum total Ig level compared to control and other experimental groups supplemented with different prebiotics (FOS and inulin) and according to these results, it has been reported that GOS may be the most suitable prebiotic. Very recently, Pour *et al.* (2023) reported that the 2% level of GOS produced the best results in terms of final weight, feed conversion ratio and body protein content in an 8-week trial on juvenile common carp with different GOS levels (1%, 2% and 4%). Ziółkowska *et al.* (2021) investigated the effects of 1% and 2% dietary supplementation of TOS produced by

enzymatic transgalactosylation from milk lactose using *Bifidobacterium bifidum* cells on tissue mineral content in common carp, and they reported that Fe concentration in skeleton, gills, meat and Zn concentration in meat of the experimental groups were significantly higher than the control group. It was also reported that there was nonsignificant difference between the groups in terms of Ca and P concentrations in the relevant tissues, but 2% TOS supplementation to the diet created a positive correlation between Mg and P in meat and skeleton, between Fe and Ca in gills, and between Fe and Zn in skeleton. It was reported by the same researchers (Ziółkowska *et al*., 2020) that the addition of TOS to the diet had no significant effect on growth performance, but had positive effects on intestinal development, significantly increased villus height, width and surface, and that the addition of 1% TOS to the diet significantly increased P absorption from the intestine compared to control and 2% TOS group.

Mannanoligosaccharides (MOS)

MOS, an indigestible short-chain polymer obtained by hydrolysis of glucomannan and galactomannan, is commercially derived from the cell wall of the yeast *Saccharomyces cerevisiae* (Wee *et al*., 2024). The wall of this yeast cell is composed of approximately 80-90% polysaccharides, 10-20% proteins, and the outermost layer contains 25-30% MOS complexed with mannoproteins followed by 30-60% β-glucan, while the innermost layer contains 1-15% chitin (del Valle *et al*., 2023). In addition to α-mannooligosaccharides obtained by chemical hydrolysis of mannan extracted from yeast cell wall, β-mannooligosaccharides can also be produced by trans-mannosylation of mannose, obtained by treatment of seed extracts such as locust bean gum, konjac gum, guar gum, and industrial by-products such as palm kernel cake, copra meal, spent coffee grounds, with the endo- β-mannanase enzyme in A.oryzae, A. niger, Bacillus spp. or complete hydrolysis with endo-β-mannanase, β-mannosidase and α-galactosidase enzymes (Kango *et al*., 2022).

The first reason for the use of MOS as a feed additive is that it prevents the adhesion of pathogenic bacteria to intestinal cells and another is to stimulate the immune system and increase the immunological effect. Yeast cell wall has a strong antigenic stimulation property and it is known that the addition of *Saccharomyces cerevisiae* to the feed (0.5-2%) strengthens the microflora and natural defense system in the intestines (Genç *et al*., 2011). The mechanisms of action of MOS in aquaculture can be summarized as follows (Ateş, 2009): (a) By the terminal mannose units in its structure, MOS forms strong bonds with the attachment sites of pathogenic bacteria to the small intestine, known as fimbriae, which contain lectins, and allows them to be excreted in the feces without harming the animal, (b) Mannans, which are found in the upper layers of the yeast cell wall and contain unique adhesion surfaces for bacteria, also

ensure optimal thickness of the intestinal wall and increase the absorption and usefulness of nutrients, (c) While mannans bind to pathogens in the intestine and inhibit their colonizing, they also create a suitable nutrient environment for *lactobacilli* and help the development of beneficial bacterial populations, (d) The proliferation of beneficial bacteria in the intestines reduces the pH of the environment and prevents the proliferation of pathogenic bacteria, (e) Since MOS is richer in carbohydrates, it acts as a trap for pathogenic bacteria that bind to existing carbohydrates in the digestive tract epithelium with surface proteins called lectins, and prevents binding of pathogenic bacteria to intestinal epithelium by binding to lectins earlier and blocking them, (f) MOS stimulates the immune system and creates strong immunity in animals, (g) MOS can bind mycotoxins in feed and render them harmless.

The results obtained in studies conducted with MOS in fish may vary depending on the level of MOS used. In a study on milkfish (*Chanos chanos*), body weight gain, FI, PI (ptotein intake), specific growth rate (specific growth rate), feed conversion ratio, protein efficiency ratio and survival rate were significantly higher in groups supplemented with 2 and 3 g MOS per kg diet compared to group supplemented with 1 g MOS and control (Harikrishnan *et al*., 2023). In the same study, the activity of superoxide dismutase (superoxide dismutase) and catalase in terms of antioxidant enzymes and phagocytic activity, respiratory burst activity and lysozyme activity in terms of immune parameters increased due to the increase in the amount of MOS added to the diet and this effect was more remarkable in the groups supplemented with 2 and 3 g MOS compared to the other groups. MOS supplementation also had a modulating effect on intestinal microflora and the group supplemented with 3 g MOS had the highest population of *Lactobacillus*. In addition, the effect of MOS supplementation against *Vibrio anguillarum* infection was also observed in their study and the mortality rate was 15, 10 and 5% for the groups supplemented with 1, 2 and 3 g MOS, respectively, while this rate was 75% in the control. In a 60-day study conducted by Dawood *et al*. (2020) in red sea bream (*Pagrus major*), 0.05, 0.1, 0.15 and 0.2% MOS were added to the diet of the experimental groups and performance parameters were significantly higher than control group, and the groups supplemented 0.15 and 0.2% MOS had better results for feed conversion ratio. There was no significant difference between the groups in terms of reactive oxygen metabolites in the evaluation of oxidative status, but except for the 0.05% MOS group, the other experimental groups had significantly higher biological antioxidant potential than control. In addition, the addition of MOS to the diet increased the tolerance to low salinity stress in the experimental groups. Especially in 0.1%, 0.15% and 0.2% MOS supplemented groups, time to 50% mortality was significantly higher than the control group. Zhu *et al*. (2023) reported that 0.2% MOS supplementation to the diet for 4 weeks increased weight gain rate and specific growth rate in terms of

performance, glutathione peroxidase, catalase, superoxide dismutase activities in terms of antioxidant activities, serum lysozyme, alkaline phosphatase, albumin and total protein concentrations in terms of non-specific immunity activities, villi length, villi width and muscle thickness measurements in terms of intestinal morphology increased significantly compared to the control group in juvenile hybrid groupers (Epinephelus fuscoguttatus♀× Epinephelus lanceolatus♂). Genç *et al*. (2020) investigated the effects of two different levels (0.1% and 0.2%) of MOS on African catfish (*Clarias gariepinus*) and reported that the addition of MOS produced significantly better results in terms of final weight, weight gain, daily weight gain and feed conversion ratio compared to the control group, only 0.2% MOS group was significantly higher than the control group in terms of survival rate, and there was no significant difference between the groups in terms of specific growth rate. In the same study, the highest villus length in terms of small intestine villi length was observed in 0.2% MOS group. Differently, in a very recent 60-day study (Nakhei *et al*., 2023) on raibow trout, it was reported that the use of MOS and β-glucan mixture at 2 ppt level had no significant effect on weight, specific growth rate, feed conversion ratio, average length parameters in terms of performance and lysozyme, total serum antibody, IgM levels, blood lymphocyte and neutrophil counts in terms of immunoassay results, but complement activity was significantly higher than the control group at the end of the experiment. In the same study, it was also determined that MOS and β-glucan mixture did not cause a significant difference in mortality against temperature stress (20°C), anoxia, salinity stress and formalin stress.

Chitooligosaccharides (COS)

It was developed as an oligosaccharide form of low solubility chitosan obtained by deacetylating chitin, a non-toxic, bioavailable biopolymer naturally found in arthropods (Jeon *et al*., 2000). However, in addition to arthropods, COS can also be obtained from the exoskeleton of insects and the cell wall of fungi by various methods such as acidic hydrolysis, oxidative degradation, microwave, γ-irradiation and enzymatic hydrolysis (Liu *et al*., 2019*a*). Although it has important functional activities, its high molecular weight and low solubility at pH values higher than 6.8 limit the practical use of chitosan (Seo *et al*., 2007). On the other hand, studies have focused on COS rather than chitosan due to its short chain length and low viscosity at neutral pH in addition to its rapid solubility in water due to free amino groups in D-glycosamine units (Kim & Rajapakse, 2005).

Studies on COS with different animal species in the past years have shown positive results. For example, improving growth performance in broilers (Huang *et al*., 2005; Li *et al*., 2007) and pigs (Liu *et al*., 2008), increasing immunoglobulin concentration and antibody titer in broilers (Huang *et al*., 2007), increasing monocyte, interleukin (IL)-6 and interferon (IFN)-γ levels

by stimulating the immune mechanism in mice infected with *S. aureus* (Moon *et al*., 2007) can be shown as remarkable results in this context. The majority of studies on the immunologic effects of COS have focused on innate immunity. It has been reported that COS shows immunomodulatory effects through recognition of COS by macrophages, which play a key role in inflammation and host defense system and are the most important component in innate immunity, and are effective in regulating their functions (Jeong *et al*., 2000; Vasconcelos *et al*., 2013). In a recent study conducted by Ouyang *et al*. (2021) on blunt snout bream (*Megalobrama amblycephala*) to reveal the mechanism behind the stimulatory effect of COS on macrophages, it was uncovered that the activation of blunt snout bream macrophages by COS relied on an internalization mechanism and a transduction pathway. It was also highlighted that mannose receptor C-type lectin-like domain 4-8 (MR CTLD4-8) was a key component in the recognition, binding, transduction of COS, and the immunoregulation of macrophages, and that MR CTLD4-8 cooperated with Toll-like receptor 4 (TLR4) in the regulation of the pro-inflammatory response of macrophages. *In vitro* and *in vivo* studies were also conducted by Ouyang *et al*. (2023). In *in vitro* study, it was found that COS reversed the immunosuppression induced by cortisol in blunt snout bream macrophages, promoted anti-inflammatory gene expressions of tumor necrosis factor (TNF)-α, IL-1β, inducible nitric oxide synthase and the production of nitric oxide and increased the phagocytic activity of macrophages. In the *in vivo* trial, the researchers noted that orally administered COS was absorbed directly through the intestine and significantly improved innate immunity by correcting cortisol-induced immunosuppression, and reported that the facilitation of gene expression of inflammatory cytokines (TNF-α, IL-1β, IL-6) and pattern recognition receptors (TLR4, MR) and increased bacterial clearance by COS were effective in healing tissue damage and survival.

Considering the functional and biological effects of COS, the effects of this prebiotic on growth performance, immunity, stress and disease resistance are still of interest in aquaculture. In a trial in koi fish (*Cyprinus carpio koi*) (Lin *et al*, 2012*a*), the addition of 0.2% COS to the diet resulted in a significant increase in final body weight and specific growth rate and a significant improvement in feed conversion ratio in the experimental group compared to the control, and also, the immunological evaluation showed a significant increase in total leukocyte count, respiratory burst activity, phagocytic activity, lysozyme activity, serum superoxide dismutase activity and a significant increase in protection against intraperitoneal injection of 0.3 ml PBS containing *A. veronii* and a decrease in mortality rate. In ovate pompano (*Trachinotus ovatus*), a common marine fish in South China, a significant increase in final body weight and a significant improvement in specific growth rate and feed conversion ratio were observed in groups supplemented with 0.2, 0.4 and 0.6%

COS compared to the control group (Lin *et al*., 2012*b*). In the same study, total leukocyte count, neutrophil, lymphocyte and monocyte percentages, respiratory burst activities, macrophage phagocytic capacities, lysozyme and superoxide dismutase activities of the COS supplemented groups were found to be significantly higher than control, and researchers reported that the addition of 0.4% COS to the diet for 56 days significantly improved growth performance, survival rate and immune response. Similarly, the use of 0.2% COS in largemouth bass (*Micropterus salmoides*) diets (Lin *et al*., 2017) and 0.2%, 0.4% and 0.8% COS in Nile tilapia (*Oreochromis niloticus*) diets (Meng *et al*., 2017) was reported to significantly improve growth performance (final weight, specific growth rate, feed conversion ratio) and immunological parameters (phagocytic activity, lysozyme activity and protection against A. *hydrophila* infection). In a recent 8-week trial on Nile tilapia (Oreochromis niloticus) with diets containing 0.4, 0.8 and 1.2% COS (Nurmalasari *et al*, 2022), weight gain, feed efficiency and specific growth rate of COS-treated groups were significantly higher than the control group. Also, 0.8% and 1.2% COS-treated groups exhibited significantly lower mortality than the other groups against infection with *Streptococcus iniae*, and phagocytotic activity, respiratory burst activity, superoxide dismutase activity, lysozyme activity and TNF-α, IL-1β and IL-8 expression were more prominent than than other groups.

Xylooligosaccharides (XOS)

XOS are sugar oligomers composed of xylose units found in nature in natural products such as bamboo shoots, fruits, vegetables, milk, honey. These oligosaccharides can be produced by various methods such as enzyme treatment of xylan-containing lignocellulosic materials, subsequent enzymatic hydrolysis of xylan isolated by chemical fractionation of a suitable lignocellulosic material or hydrolytic degradation of xylan by steam, water or dilute mineral acid solutions (Vázquez *et al*., 2000). The chemical structure of XOS, which generally consists of xylose chains linked by β-(1-4) bonds with a degree of polymerization ranging from 2-10, can vary depending on the xylan source, and studies on mammals with these oligosaccharides have shown that they increase the activity of beneficial intestinal bacteria such as *Bifidobacterium*, which leads to an increase in short-chain fatty acids (SCFAs) in the secum, and it has shown benefits such as improved intestinal function, mineral absorption, lipid and glucose metabolism, immune modulation, anti-oxidant, anti-inflammatory and anti-microbial functions (Aachary & Prapulla, 2011; Broekaert *et al.* , 2011). Studies on XOS have mostly focused on mammals and studies on aquatic species are not yet sufficient. However, as mentioned above, in addition to the beneficial effects of prebiotics such as fructooligosaccharides, mananoligosaccharides and chitooligosaccharides on aquatic species, XOS has also attracted attention as a promising prebiotic in aquaculture.

In a 7-week experiment on glucose and lipid metabolism in European sea bass (*Dicentrarchus labrax*) juveniles (Guerreiro *et al*., 2015), 1% XOS was added to each of diets containing two different protein sources (fish meal or plant protein). As a result of the experiment, it was determined that the growth performance of the group fed with plant protein diet supplemented with XOS was higher than the control group fed with plant protein diet. Glucokinase activity was higher in the XOS supplemented fish meal diet group than in the control group fed fish meal diet. However, regardless of the dietary protein type, XOS supplementation to the diet decreased lipogenesis by decreasing the activities of lipogenic enzymes. Based on these results, the researchers reported that XOS has good potential as a prebiotic. The positive effects of XOS on lipid metabolism are important findings in terms of fish health and survival of the flock. As a matter of fact, feeding high-fat diet for a long period of time leads to high mortality rate, decreased growth performance, excess fat, and excess fat storage in the liver (Lu *et al*., 2013). In this context, Abasubong *et al*. (2018) drew attention to the positive effects of XOS on lipid metabolism in common carp (Cyprinus carpio) fed a high-fat diet, and as a result of the 8-week experiment, plasma cholesterol, triglycerides, LDL levels were lower and HDL levels were higher in groups fed diet supplemented 1% and 2% XOS, and they also noted a decrease in the transcription of lipoprotein lipase, whereas the transcription of carnitine palmitoyltransferase I, peroxisome proliferator-activated receptor alpha, acyl-CoA oxidase, and CD36 was increased. Recently, to demonstrate the relationship between prebiotics and carbohydrate metabolism, Chen *et al*. (2022) conducted a 12-week study in which XOS was incorporated into the diet of blunt snout bream (*Megalobrama amblycephala*) that were fed a high carbohydrate diet. They found that nitrogen retention efficiency, liver glycogen contents, the transcriptions of glucose transporter 2, glucokinase, pyruvate kinase, glucose-6-phosphatase, carnitinepalmitoyl transferase 1 and acyl-CoA oxidase levels were significantly higher in the 1% XOS group compared to the group fed high carbohydrate diet. In addition, as a result, they reported that the addition of 1% XOS to the diet in the group fed high carbohydrate diet had an ameliorative effect on growth performance and glycolipid metabolism and this was realized by up-regulatory effect on glucose transport, glycolysis, glycogenesis, the pentose phosphate pathway and fatty acids β-oxidation and down-regulatory effect on gluconeogenesis and fatty acid biosynthesis.

There are also studies investigating the effects of XOS on growth performance, immunity, antioxidant capacity and disease resistance in fish. In a 60-day trial on Grass carp (Ctenopharyngodon idella), the addition of 0.002%, 0.004%, 0.006%, 0.008% and 0.010% of XOS to the diet significantly improved final body weight, specific growth rate and feed intake compared to the control group, and especially the addition of 0.004% XOS significantly improved percentage of weight gain and feed efficiency (FE) compared to the

control group (Sun *et al*., 2021). In the same study, positive results were obtained in terms of gut microbiota and XOS supplementation to the diet significantly decreased the number of *Aeromonas* and *Escherichia coli* and significantly increased the number of *Lactobacillus* and *Bifidobacterium*. In an 8-week study on the same fish species, Zhang *et al*. (2020) reported that growth performance values of 0.05%, 0.1%, 0.2%, 0.4% and 0.6% XOS groups were significantly higher than the control group and the highest final body weight, weigth gain and specific growth rate values were observed in the group given 0.1% XOS. In terms of plasma immune parameters, dietary XOS supplementation increased plasma lysozyme activity and decreased malondialdehyde activity. The highest lysozyme activity and the lowest malondialdehyde activity in plasma and the highest superoxide dismutase activity in liver were detected in the group supplemented with 0.1% XOS. In addition, the highest survival rate against *A. hydrophila* was detected in the 0.1% XOS group and it was reported that the most appropriate level of XOS addition to the diet was 0.1%. In a 56-day trial conducted by Wang *et al*. (2022) in rainbow trout (*Oncorhynchus mykiss*) in which dietary XOS levels were 0.25%, 0.5%, 0.75% and 1%, the best result in terms of weight gain was found in the 1% XOS group, and in terms of immune-related genes, it was observed that the addition of 0.75% and 1% XOS to the diet significantly decreased intestinal TNF-α and IL-6 gene expression and inhibited the production of these cytokines, which cause damage to the tight junction structure of intestinal epithelial cells. Considering the negative effects of excessive fat in fish diets on fish (as mentioned above), the effects of XOS addition to diets on performance, antioxidant and immune parameters in a trial conducted by Abasubong *et al*. (2022) on common carp (*Cyprinus carpio*) fed a high-fat diet are noteworthy in this context. In their experiment, 0.5%, 1%, 2% and 3% XOS were added to the high-fat diet for 56 days, and as a result of the experiment, it was reported that the addition of 1, 2 and 3% XOS to the diet was effective in eliminating the damages of high-fat diet feeding by promoting growth, positively affecting immune parameters and improving immune status, reducing pro-inflammatory gene expression and eliminating oxidative stress.

Soybean Oligosaccharides (SBOS)

SBOS, which are important nutritional components of soybeans, are prebiotics commonly isolated from soybean seeds and have been reported to be a generally recognized safe material in the United Nations (Gatesoupe, 1999; Chen *et al*., 2010). SBOS are mainly soluble oligosaccharides known as sucrose, raffinose and stachyose, and raffinose is a trisaccharide containing galactose linked α-(1–6) to the glucose unit of sucrose; stachyose is a tetrasaccharide containing a galactose linked α-(1–6) to the terminal galactose unit of raffinose (Ma *et al*., 2017; Kim *et al*., 2003). The content of raffinose and stachyose in soybeans varies between 1-2% and 5-6%, respectively (Francis *et al*., 2001).

Although limited in number, there are also studies showing that SBOSs have positive effects on fish, as with other prebiotic oligosaccharides. In a study investigating the effects of the addition of SBOS with raffinose and stachyose contents of 0.61% and 2.61%, respectively, to fish meal-based and soy protein isolate-based juvenile Japanese flounder (Paralichthys olivaceus) diets on lipid metabolism, it was observed that there was no negative effect on lipid metabolism in the groups fed fish meal-based diet, however, it reduced the incidence of fatty liver in those fed soy isolate protein-based diet (Deng *et al*., 2007). In a 56-day experiment conducted by Sørensen *et al*., (2011) on common carp (*Cyprinus carpio* L.), the effects of dietary raffinose supplementation at 0.1, 0.2 and 0.4% on immune parameters were investigated. In their study, addition of raffinose to the diet significantly increased skin mucus lysozyme and serum lysozyme activity compared to the control group, and skin mucus total immunoglobulin level was significantly higher than the other groups only in 0.4% raffinose group. Serum total immunoglobulin levels were not affected by the supplementation of raffinose. In terms of immune related gene expression, the highest relative expression of IL-1β gene in the intestine was found in 0.1% raffinose group, relative expression of lysozyme gene in the intestine was found in 0.1% and 0.2% raffinose group, but no significant difference was found between the groups in terms of TNF-α gene expression. In conclusion, the researchers reported that the addition of 0.1% and 0.2% raffinose to the diet supported immune competence and health in common carp. According to a study performed by Xu *et al*. (2018) to investigate the effects of dietary raffinose supplementation on growth, non-specific immunity, intestinal morphology and microbiome in juvenile hybrid sturgeon (Acipenser baeri Brandt ♀ × A. schrenckii Brandt ♂), 0.1% raffinose addition to the diet caused a significant increase in final body weight, specific growth rate and weight gain ratio, and although it did not show a significant effect on serum lysozyme, malondialdehyde, superoxide dismutase, complement 3 and 4 levels in terms of immunological and antioxidant parameters, it significantly increased serum myeloperoxidase and respiratory burst activity of phagocytes, which is a part of non-specific immunity. Also, the addition of raffinose to the diet positively affected intestinal health by increasing the density of intestinal mucosal folds and microvilli in the fish and caused the formation of a smoother and more uniform intestinal wall than the control group. As a result, the researchers reported that raffinose supplementation to the sturgeon diet improved growth performance by having a regulatory effect on intestinal histology and microbiota and that raffinose can be considered as a useful supplement on growth and health in sturgeon fish. Abdel-Latif *et al*. (2020) tried to determine the optimum raffinose level in Nile tilapia (*Oreochromis niloticus*) in terms of growth performance, oxidative status and immunity. The optimal level of raffinose was determined as 1.23, 1.37, 0.85 and 1.01 g/kg diet on the basis of specific growth rate, feed conversion ratio, superoxide dismutase

measurement and lysozyme activity, respectively, and when all measurements were taken into consideration, they recommended that the optimum raffinose level should be between 0.85-1.37 g/kg diet in Nile tilapia.

The different results obtained in studies do not seem to fully clarify whether stachyose has positive effects as a prebiotic. Indeed, although some researchers have reported that stachyose has no prebiotic effect on Japanese flounder (Mi *et al*., 2011), Atlantic salmon (Sørensen *et al*., 2011) acting as an anti-nutritional factor and negatively affects performance and feed utilization on silver crucian carp (Cai *et al*., 2012), some researchers have reported that this oligosaccharide can improve intestinal health by selectively feeding beneficial bacteria such as *Bifidobacterium* and *Lactobacilli* in mice (Li *et al*., 2013), human (Li *et al*, 2017) and in vitro, and has positive effects on intestinal and body health by increasing the production of short-chain fatty acids (SCFAs) and other nutrients beneficial to the immune system (Mussatto & Mancilha, 2007). Several recent studies on fish have shown promising results that stachyose can be added to the diet as a prebiotic. Yang *et al*. (2018) investigated the effects of 0%, 1.25% and 5% stachyose supplementation on intestinal microbiota profile and intestinal mucosal barrier function in a 12-week experiment on juvenile turbot (*Scophthalmus maximus* L.). The researchers reported that the addition of 1.25% and 5% stachyose to the diet may have ameliorative effects on intestinal cellulolytic bacteria and barrier-forming tight junction proteins gene expression and increase mucosal barrier function in the intestines. They also stated that especially 5% stachyose has a highly regulating effect on intestinal microbiota, but it should not be ignored that it increases the abundance of potentially pathogenic bacteria along with beneficial bacteria. In the study conducted by Dai *et al*. (2021) in the same fish species, the addition of 25.0 g/kg stachyose to the diet did not cause a significant difference in wight gain, specific growth rate, feed efficiency and feed intake as performance parameters, but complement 3 and 4, immunoglobulin M and lysozyme levels as serum immune parameters were significantly higher than the control group. Furthermore, addition of stachyose to the diet improved intestinal mucosal barrier function, increased the abundance of *Lactobacillus* and *Bacteroides* and decreased the abundance of *Mycoplasma*, a potential pathogen, compared to the control group.

Apart from the use of SBOS as feed additives, a very recent study investigating their effects in the biofloc system has presented remarkable results in this regard. Biofloc Technology is known as a technology that allows continuous conversion and repeated use of nutrients and minimal water exchange, allowing the proliferation of beneficial microorganisms in the environment and the protection of water quality by converting nitrogenous compounds accumulated in the environment where fish are cultured into microbial protein, and increasing profitability by reducing feed costs in

terms of fish nutrition (Emerenciano *et al*., 2013). The aim of this technology, which aims to establish a carbon/nitrogen balance in aquaculture systems, is to increase the number of nitrogen-utilizing bacteria by adding extra carbon to the environment (either through a carbon source or through the addition of feed with increased carbon content), thereby, to reduce the concentration of ammonium in the water and to improve water quality (Hargreaves, 2006). In this context, Qui *et al*. (2023) carried out a study in which glucose was replaced with SBOS by 1, 5, 10, and 100 % to investigate the effects of using SBOS instead of glucose as carbon source on crucian carp cultured in biofloc system, and as a result of the study, 1% SBOS improved growth performance, digestion and anitoxidant capacity in fish, 5-10% SBOS effectively reduced intestinal *Pseudomonas* and *Vibrio* abundance in fish, and it was reported that the optimal replacement ratio for SBOS is 1-5%.

Alginate Oligosaccharides (AOS)

Alginates, a natural polyuronic saccharide, are mainly produced by brown and red algae and some bacteria, but the majority of alginates are now commercially extracted from *Phaeophyta* plants such as *Ascophyllum*, *Durvillaea*, *Ecklonia*, *Lessonia trabecculata*, *Macrocystis*, *Sargassum* (Westermeier *et al*., 2012). *Laminaria hyperborean, Macrocystis pyrifera, Laminaria digitata, Ascophyllum nodosum, Sargassum spp., Laminaria japonica* as algae, and *Azotobacter vinelandii* and *Pseudomonas spp*. as bacteria. can be counted as some alginate sources (Liu *et al*., 2019b). Alginate, a kind of acidic linear polysaccharide composed of alpha-L-guluronate and its C-5 epimer beta-D mannuronate linked by 1,4-O-glycosidic bonds, exists in three forms: poly-alpha-L-guluronate (pG), polybeta-D-mannuronate (pM) and heteropolymeric regions (pMG) (Zhu *et al*., 2016). Poly-alpha-L-guluronate-rich alginates have higher water solubility than polybeta-D-mannuronate-rich alginates (Jimenez-Escrig & Sanchez-Muniz, 2000). Alginate oligosaccharides (AOS) are oligomers consisting of 2-25 monomers (Liu *et al*., 2019b). Due to its low molecular weight, non-toxic, non-toxic, biocompatible and degradable polymer, AOS has found applications in various fields such as production of plants and microorganisms, use as cryoprotector, feed additive and prebiotic in aquaculture, poultry and pig nutrition (Liu *et al*, 2019b; Yang *et al*., 2021), these oligomers have antioxidant, antimicrobial, antiinflammatory and immunomodulatory effects in terms of bioactivity (Liu *et al*., 2021).

Although feeding studies on various fish species such as Atlantic salmon, tilapia, sea bream, Malaysian mahseer, Asian seabass (Gabrielsen *et al*., 1998; Merrifield *et al*., 2011; Peso-Echarri *et al*., 2012; Van Doan *et al*., 2016; Asaduzzaman *et al*., 2019; Ashouri *et al*., 2020) have been conducted on alginates in the field of fish farming, it is noteworthy that there are very few studies on AOS, but it is seen that it is an oligosaccharide that has attracted interest in recent years. In a 6-week experiment conducted by Yang *et al*. (2021)

to investigate he effects of 0.5%, 1.0% and 2.0% AOS addition to the diet on liver fat metabolism and antioxidant capacity in grass carp (*Ctenopharyngodon idellus*), body weight and changes in wight values of the groups given AOS were significantly higher than the control, and feed conversion ratio improved significantly. In this experiment, in which the group given 1.0% AOS showed the highest weight gain, the differences in codition factor index and splenic/liver somatic indices were nonsignificant. Dietary AOS supplementation significantly reduced cell vacuolization and fat accumulation in the liver in a dose-dependent manner. In the same study, protection against *A. hydrophila* infection was also evaluated and it was found that AOS supplementation significantly decreased the number of necrotic and vacuolized cells in the head kidney and liver in a dose-dependent manner compared to the control group. In addition, superoxide dismutase, catalase and glutathione peroxidase enzyme activities evaluated in terms of liver antioxidant capacity were significantly higher in 1.0% and 2.0% AOS groups compared to the control group, and malondialdehyde levels measured to investigate liver oxidative damage were significantly lower in all AOS groups. Li *et al*. (2022) found that the same level of AOS supplementation to diets on the same fish species increased antioxidant gene expression and enzyme activity in the gut, increased CSF, IgM and MHC-II expression among immune factors, decreased TNF-α and IL-1β expression among pro-inflammatory cytokines, increased IL-10 expression, which is an anti-inflammatory cytokine, increased the survival rate against *A. hydrophila* infection, and also it was reported that while the relative majority of *Cetobacteria* and *Lobacteria* among intestinal probiotic bacteria significantly increased, the population of harmful bacteria such as *Fusobacteria*, *Proteobacteria* and *Verobacteria* decreased. The results reported in the experiment carried out by Hu *et al*. (2021), in which similar effects in terms of performance and non-specific immunity were obtained in the same fish species with the addition of lower concentrations of AOS, are noteworthy in this respect. In their study in which 100, 200 and 400 mg of AOS were added per kg dry diet, there was a significant improvement in final weight, body weight gain percentage, feed conversion ratio, specific growth rate and survival rate values among growth performance parameters, a significant increase in superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and decrease malondialdehyde levels among serum antioxidant parameters, significant increase in total protein, lysozyme, alkaline phosphatase, complemett 3 and 4 levels among non-specific immune parameters, a significant decrease in IL-1β, IL-8, TNF-α gene expression among pro-inflammatory cytokines and a significant increase in IL-10 gene expression among anti-inflammatory cytokines in the AOS supplemented groups compared to the control, and it was reported that the optimum dose in terms of the related parameters was 200 mg/kg among the groups given AOS. As a different species of fish, in a 56-day trial conducted by Huang *et al*. (2022) on *Trachinotus ovatus*, the addition of 0.7

and 6. 0 g/kg of AOS in the diet caused an increase in plasma immune indexes and hepatic antioxidative capacity, inhibition of intestinal nuclear factor kappa B (Nf-κb) and proinflammatory cytokine mRNA expression, improvement in antiinflammatory cytokine mRNA expression and increase in intestinal villus height, and reported that the optimum level of AOS was 0.7 g/kg. The study on Atlantic salmon by Gupta *et al.* (2019), which concluded that low levels of AOS are more effective than high levels in terms of intestinal microbiota, is noteworthy in this respect. In their 9-week trial with 0.5% and 2.5% addition of microalga (*Laminaria sp.*) derived AOS to the diet, although the addition of 2.5% AOS reduced the overall intestinal bacterial diversity, the addition of 0.5% AOS did not cause a decrease in diversity and allowed bacteria such as *Brevinema andersonii*, *Aliivibrio logei*, *A. parvum*, *A. insolitus*, which have the capacity to degrade carbohydrates and produce butyric acid, to predominate within this diversity, creating a better prebiotic effect for animal health.

Conclusion

In this chapter, various effects of prebiotic oligosaccharides used as feed additives in fish farming are mentioned. Studies show that these oligosaccharides have positive effects on growth performance, immune system, antioxidant activity, gut health and microbiota of fish. The use of prebiotic oligosaccharides promotes the proliferation of beneficial bacteria by balancing the gut microbiota of fish. This improves the health of the digestive system and positively affects the general health of the fish by increasing resistance to pathogens. In addition, the antioxidant activities of oligosaccharides support fish to be more resistant to stress factors by reducing oxidative stress at the cellular level. Prebiotic oligosaccharides, which strengthen the immune system of fish, reduce mortality rates by increasing resistance to diseases. The use of these substances as feed additives also offers significant economic advantages by increasing growth performance and feed efficiency. Future studies on prebiotic oligosaccharides, which have positive effects on fish health and production efficiency, will help to better understand their long-term effects and possible side effects by determining the most effective dosages and combinations of these feed additives and will contribute to sustainable and nature-friendly aquaculture practices.

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CHAPTER 4

GENERAL APPROACHES TO MASTITIS IN CATTLE

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1. Mastitis in cattle and its causes

Bovine mastitis is the most prevalent condition affecting dairy cattle, a mammary gland infection that lowers milk quality and quantity, resulting in financial losses. Numerous gram-positive and gram-negative bacteria are examples of etiological agents. They may be environmental (e.g., *Escherichia coli*, *Enterococcus* spp. *Streptococcus uberis*) or contagious (e.g., Staphylococcus aureus, Streptococcus agalactiae, Mycoplasma spp.) (Cheng & Han, 2020).

Mastitis in cattle is classified as clinical, subclinical, or chronic based on the level of inflammation. There are three types of clinical mastitis: acute, acute, and subacute (Kamel & Bakry, 2020).

1.1. Epidemiology

The development of mastitis depends on the animal (host), the infection (causative agent), and the environment in which the animal lives. The breed, the mammary gland's physiological state, and the teat duct's anatomy, sphincter tone, and teat structure are essential among the host factors. The pathogen's immediate surroundings, its capacity to colonize the teat canal, its ability to stick to the teat, and its capacity for survival are all included in the pathogen factor. Environmental factors, housing system, the condition of the place where it lies, milking system, correct milking, and milking hygiene are effective (Makovec & Ruegg, 2003).

1.2. Host response and pathogen transmission

The nipple is the breast's initial line of defense against infections. A sphincter made of smooth muscles opens and closes it, serving as a barrier to keep milk from leaving and infections from entering the duct. The stratified squamous epithelium lining the mammary duct forms keratin, which fills the duct between 30 and 2 hours after milking. This time interval can vary, allowing bacteria near the opening to enter the nipple duct. Fatty acids and fibrous proteins make up keratin. The fibrous proteins attach electrostatically to the pathogens once they enter the mammary duct, altering their cell walls and increasing their sensitivity to osmotic pressure. Failure to maintain osmotic pressure results in cell membranes' breakdown and invading pathogens' death. A number of distinct physical and physicochemical parameters, such as the nipple duct's length and width, the amount of keratin present, and the milking ability, as shown by the peak flow rate, all affect how well the nipple defends against infections. The illness process in the mammary gland is caused by bacterial infections that can enter through the duct opening and evade keratin's antibacterial properties. (Cheng & Han, 2020; Cobirka et al., 2000).

The host's immune response, which is the second line of protection, must then react. He stated that the host's immunological response and genetic

makeup mainly determine the disease's severity, but bacterial strains' virulence can also significantly impact the disease's severity. The main pathogen-related factors include the bacterial vaccine's species, virulence, strain, and size. In contrast, host factors include parity, lactation stage, age, animal immune status, stress, and vaccination status (Chauhan et al., 2000).

Cell surface receptors that can identify microbial compounds and soluble elements like acute phase proteins and cytokines released into the blood and milk are essential for the immune system's reaction to the early phases of infection. The host's reaction varies depending on the kind of substance that causes mastitis. Lactate dehydrogenase activity and immunoglobulin G concentrations are significantly higher in gram-negative bacteria, like E. coli, than in gram-positive bacteria. This implies that, when combined with SCC, lactate dehydrogenase can be utilized as a marker to differentiate between Gram-positive and Gram-negative bacteria (Hessle et al., 2020).

Antibiotics are currently the primary treatment for mastitis, however glucocorticoids like prednisolone are also included in these formulations. They aid the endangered host organism in restoring the lower milk quality brought on by mastitis and strengthening the blood-milk barrier. More significantly, prednisolone can influence the recruitment of immune cells to inflammatory regions by binding to the glucocorticoid receptor on cells and inhibiting the synthesis of pro-inflammatory cytokines. Prednisolone may potentially have positive and negative effects on mastitis treatment because it also weakens the mammary gland's immune system by lowering the amount of immunological globulin G in the milk. They found that supraphysiological doses of oxytocin opened the blood-milk barrier and strengthened patency during lipopolysaccharide-induced mastitis. According to these results, oxytocin can be utilized effectively when there is little to no immune-globulin G transfer because it causes the more significant transfer of the blood component into milk. This frequently happens when mastitis is asymptomatic (Yamini, 2020).

A pathogen must survive and proliferate to have a pathogenic effect, in addition to entering the mammary gland, for inflammation to ensue. As animals vary in their resistance to microbial entry into the mammary gland and their reaction to resolving inflammation, so too may the virulence of the pathogens that cause mastitis (Rainard et al., 2020).

1.3. Pathogenesis

Methods of detection: Creating a suitable treatment requires a thorough grasp of mastitis's pathogenicity. Although various bacterial strains are the leading cause of mastitis, reports of viral, algal, and fungal mastitis have also been made (Eriksson, 2005).

It occurs mainly through the mammary duct, except in cases of mastitis, tuberculosis, leptospirosis, and brucellosis, where the spread method may be hematogenous. Invasion, infection, and inflammation are the three phases that make up the development of mastitis. The time pathogens travel from the nipple via the mammary duct and into the milk is known as the invasion stage. The infection stage is when pathogens multiply rapidly and invade the breast tissue. In addition to mild to acute systemic effects, there are gross and subclinical abnormalities in the breast and milk. Different clinical problems exist throughout the inflammatory stage (Radostit et al., 2007).

Inflammation is triggered when germs infect the mammary gland. These bacteria proliferate and generate enzymes, poisons, and components of their cell walls. Inflammatory cells produce a variety of mediators of inflammation in response to stimulation by the body's second line of defense. The causal pathogen, age, lactation stage, cow immunological condition, genetics, and nutritional status can all affect the inflammatory response (Harmon, 1994).

Polymorphonuclear neutrophil (PMN) leukocytes and phagocytes migrate from the bone marrow to the brain, and numerous chemical messengers (chemotactic chemicals) from invasive and injured bacteria attract tissues. PMN masses can pass between milk-producing cells into the alveolar lumen, thereby increasing the amount of milk and damaging secretory cells and somatic cell count (SCC). The organisms surround the PMN bacteria at the site of infection and secrete enzymes that can destroy the bacteria. Additionally, leukocytes in the milk may release certain chemicals that draw additional leukocytes to the area. There are still a lot of somatic cells after the germs have been eliminated. A buildup of leukocytes and blood clotting factors can bring on blood clots. Complete removal of milk and small ducts is impossible due to damage to the epithelial cells and occlusion of the ducts. In certain instances, this causes scar tissue to form and the gland's affected area to permanently stop functioning. In other situations, inflammation may go down during nursing or breastfeeding, tissue healing may take place, and function may be restored (Vlasova, 1994).

1.4. Prevention-control

Eliminating the conditions that expose the teats to bacteria and reducing the likelihood of cow-to-cow spread are the most effective ways to prevent new infections (Petersson-Wolfe et al., 2010).

1.4. Hygienic procedures

Millers should always wear gloves, which should be changed and disinfected frequently. Before attaching the milking heads, 5ml of milk should be taken from each teat and examined for changes (Setlhare, 2016). Before milking, the dirt on the teat should be cleaned with a disposable towel.

In addition, disinfectants can also be used. After this cleaning, we should make sure that the teats are dry. If there is wetness in these areas, we can cause an increase in pathogens that will cause mastitis. In addition to these applications, we can apply pre-dipping before milking. We should apply this pre-dipping process with dipping cups on each teat for 30 seconds. We should use a towel for each cow. Teat tips cracking, cracks, or lesions cause mastitis; we should check the presence of these, and if there are such problems, we should take precautions. The last thing to be done is the final dipping of the teats after milking to prevent contamination in the external environment (Singh et al., 2024).

1.4.1. Milking of infected animals

Cows infected with mastitis or treated with antibiotics should be milked last or milked separately. Failure to separate infected cows and milking with others is a significant risk factor (Abebe et al., 2023).

1.4.2. Milking equipment

When the active substance penetrates the teat during milking, infection may occur in the organism. Due to irregular vacuuming, milk may flow back to the nipple and milk may be left in the udder that is not thoroughly milked. Milk remaining in the mammary duct after milking may also cause mastitis (Abebe et al., 2023; Petersson-Wolfe et al., 2010)

The milking heads must not be removed suddenly. The heads should remain attached until the vacuuming is switched off. 10-15% of the infections occur due to incomplete milking, insufficient vacuuming, and sudden removal of the milking heads. Regular maintenance of the milking machine is required. Vacuuming, voltage adjustment, etc. Before maintenance, these should be measured during milking, and corrections should be made. Monthly rubbers and nipples should be changed (Paliy et al., 2020).

1.4.3. Vaccination

One potential treatment for mastitis in cattle is vaccination. Most vaccines protect against *E. coli*, *S. aureus*, and *S. agalactiae*. The entire organism (inactivated, highly encapsulated, or non-encapsulated cells and attenuated vaccines) or subunits (toxins, bacterial surface extract, and crude extract of polysaccharides) make up *S. aureus* and *S. agalactia*; mutant core antigen J5 has been utilized extensively for *E. coli*. However, vaccines do not offer trustworthy protection (Cheng & Han, 2020).

As previously indicated, a variety of bacterial pathogens can cause mastitis. The lack of effectiveness of vaccines may be a result of the multietiological nature of bovine mastitis. The bacterial strains may differ in their virulence characteristics and immunogenic potential, in addition to the site of infection

in the mammary gland. Consequently, immunization by itself is not successful in preventing mastitis, regardless of the type of vaccine, particularly in dairy herds with high mastitis rates. Vaccination should be used in conjunction with other management measures, such as sanitary milking, antibiotic treatment, infected culling, etc., to lower the frequency and length of mastitis cases. Finding a vaccination that can protect against several strains is essential since a herd may contain multiple strains. It should also be reasonably priced and simple to incorporate into daily life (Charlier et al., 2022; Cheng & Han, 2020).

2. Mastitis treatment

The main goal of mastitis treatment is to heal the distressed udder lobe and the animal and to restore the milk yield to the previous level. For this reason, parental or intramammary antibiotics may be used. In addition to antibiotics, non-steroids, hormones, and herbal medicines can be used in treatment. The most significant advantage of the antibiotic drug given to the breast is that the active substance reaches the highest concentration in the milk and is given directly to the infected breast (Kuru & Oral, 2013).

2.1. Herbal treatments

Some plants used to treat mastitis are *Ocimum sanctum* (basil), *Panax ginseng*, *Aloe vera*, and *Symphytum offinale*. The effect of these plants was as follows: Basil plant was used in the breast, and its effect was examined. A specific increase in neutrophils and lymphocytes has been noted due to this use, and the basil plant has a decreasing effect on the overall number of microorganisms. *Panax ginseng* is used subcutaneously for 6 days at a dose of 8mg/kg once a day for subclinical mastitis, and studies have reported that this plant is beneficial in reducing the number of somatic cells. The extract of the yellow sage herb produced an anti-inflammatory effect in the intramammary application. Studies have shown that the *Symphytum offinale* is effective in acute mastitis treatments. However, it does not affect chronic cases (Kuru & Oral, 2013).

2.2. Antibiotic treatment

We often use antibiotics for treatment during the dry period. Antimicrobial treatment of dry cows is allowed among animal species as a preventative approach. For the treatment of clinical mastitis, the etiology, antibiotic susceptibility, and recommended treatment principles are essential when selecting antibiotics. According to reports, pathogens isolated from mastitis milk exhibit a wide range of drug susceptibility. The highest levels of antibiotic resistance were found in T. pyogenes infections against oxytetracycline (46.2%) and benzylpenicillin (56.3%) in a study carried out in the Zenica region of Bosnia and Herzegovina. High rates of resistance to trimethoprim-sulfamethoxazole, norfloxacin, and tetracycline were noted

despite the effectiveness of antibiotics, including florfenicol, cefoperazone, cephalexin, and ceftiofur. As a result of the emergence of antibiotic resistance, the choice is made according to the results of sensitivity tests and culture. Since antibiotics leave residues in milk, we should choose the right antibiotic. Antibiotics such as amoxicillin, oxytetracycline, and ciprofloxacin in cows caused the presence of antibiotic residues in both raw and boiled milk at different time intervals. For these reasons, antibiotics should be used strategically in mastitis cases. Combined systemic and intramammary use of antibiotics increased the cure rate. There is currently no effective option other than antibiotics. Intramammary infusion of tilmicosin may be an effective option to prevent mainly environmental streptococci and coagulase-negative staphylococci. Intramammary infusion of ceftiofurour was shown to prevent *S. uberis* infection. A comparative study of two antibiotics, tylosin base and penetrate hydriodide, showed treatment rates of mastitis; 79.8% and 82.0% of treated cows showed successful outcomes, respectively. When the use of antibiotics with non-steroidal anti-inflammatory drugs was compared with the use of antibiotics alone, reasonable, effective results were obtained when they were used together. In mastitis caused by *E. coli*, non-steroidal anti-inflammatory drugs are used as supportive in addition to antibiotics (McDougall et al., 2007).

Increased diagnostic efforts are required to prevent unnecessary antibiotic use to successfully implement antibiotic use in reducing mastitis. Given the expense of treating mastitis and its possible advantages, the scientific rationale for lowering antibiotic use, the cautious use of antibiotics with appropriate understanding, and the legal necessity for acceptable use should all be put into practice (Krömker & Leimbach, 2017).

3. Diagnosis

Changes in the udder and milk should be taken into consideration in the examination of clinical findings. In clinical mastitis cases, problems such as redness and swelling occur in the udder, in addition to pain and temperature increase. The color of the milk changes and clots can be observed. In subclinical mastitis cases, no clinical findings are observed. Tests have been developed since clinical findings are not detected and overlooked in mastitis cases. The tests measure changes in somatic cell count, enzymes, and some chemical parameters. These include the California mastitis test (CMT), strip cup, acidity, catalase, somatic cell count, electrical conductivity (EI), and milk chlorine detection. The primary purpose of the CMT test is to identify subclinical mastitis in cattle (İlhan, 2018).

4. Laboratory examinations

The diagnosis of this disease can be determined precisely by laboratory analyses. In order for this laboratory analysis to be accurate, it is essential that

the milk samples are correctly stored and kept sterile. For this purpose, the udder lobes should be cleaned with 65-70% alcohol, waited for 30-40 seconds, and the sample should be taken from the middle milking into sterile tubes and delivered in accordance with the cold chain (İlhan, 2018).

4.1. Bacterioscopy

We prepare preparations from milk samples brought to the laboratory, stain these preparations with appropriate staining methods, and examine them under a light microscope. The phenomenon we will observe in this examination is the bacterial load in milk (Yousef & Carlstrom, 2003).

Any test applied in the preliminary diagnosis of mastitis cases is insufficient. The golden rule for definitive diagnosis is the culture method. Milk samples are added to media (such as blood agar, Edwards medium, MacConkey agar, and sabouraud dextrose agar) and incubated sufficiently in suitable conditions. Using species-specific primers applied to the colonies, polymerase chain reaction (PCR) can also be used to identify the organisms developed at the end of this process (Shome et al., 2011; Yousef & Carlstrom, 2003).

4.2. Molecular methods

In determining the etiology of mastitis, techniques based on the amplification of genomic tools in vitro environments have been increasingly used in recent years. The PCR method is intensively applied among these techniques due to its advantages, such as rapid results and determining live and dead agents. With PCR, the disease can be successfully diagnosed directly in milk materials and the identification and characterization of cultured agents (İlhan, 2018; Shome et al., 2011; Yousef & Carlstrom, 2003).

5. Clinical symptoms

When mastitis strikes, the breast may become red, rigid, and heated to the touch. Some inflamed glands experience alterations in blood flow. In certain instances, the cow may even experience pain when the udder is palpated. In addition, abnormal milk from the udder may be noticed: the milk may have yellowish clots. In addition, blood may be present in the milk. The color shifts towards red (Scholte, 2019).

6. Bacterial agents causing mastitis

6.1. *Staphylococcus* spp.

The most important species of *Staphylococcus* genus bacteria causing mastitis is *S. aureus*. Apart from this species, *S. epidermidis* and *S. hyicus* species were also found to cause mastitis in cows. Bacteria of the genus *Staphylococcus* are important for mastitis: Eubacteria order, Firmicutes branch, Bacilli class, Bacillales order, and *Staphylococcaceae* family. Staphylococci, of more than 30

species, are characteristically round, Gram-positive bacteria with a diameter of 0.5-1.5 μm, often appearing as irregular clusters, sometimes as tetrads, paired cocci, or single cocci. Staphylococci are immobile, facultative aerobic, and can grow on media containing high salt concentrations (10% NaCl) and at 18-40 °C. Some of the staphylococcus species, which are immobile, spore-free, and capsule-free, may be encapsulated when they are first isolated from the organism. It is known that the structures in the cell wall of staphylococci, such as capsule, protein A, peptidoglycan layer, teichoic acid, coagulase, hemolysins, deoxyribonuclease (DNase), hyaluronidase, phosphatase, and virulence are related (Yüksekdağ & Baltacı, 2013).

They stated that S. aureus infections significantly cause mastitis in dairy cattle due to the short cure rate of medicines given during lactation. Chronic infections usually result in the culling of afflicted animals. The only effective ways to manage mastitis produced by this pathogen are to stop new infections and eliminate afflicted animals. Like other pathogenic bacteria, it spreads through washcloths, the hands of milking staff, and parts of milking machines (Cheng & Han, 2020; Cobirka et al., 2020).

Farmers frequently lament that recovery rates under in vivo settings are lower than anticipated, although *S. aureus* strains are responsive to a broad spectrum of treatments in vitro. The fact that S. aureus may endure neutrophil activity, causing udder fibrosis and infiltrating mammary epithelial cells, is likely evidence supporting this finding. However, its capacity to create microabscesses that block antibiotics from getting to the infection is the primary cause of the poor cure rate. Research indicates that staphylococcal mastitis typically results in long-term output losses. The infection reduces the cow's capacity to produce milk by permanently damaging the udder's secretory tissue, which is replaced by non-secretory tissue. Although S. aureus can spread from cow to cow, it has also been observed to survive in a particular dairy barn environment in between milkings. This pathogen is found in heifers. S. aureus is responsible for 10–12% of clinical mastitis cases. S. aureus typically lingers in the udder and responds poorly to in vivo therapy. Although penicillin antibiotic resistance is the most well-known, study years and nations have different levels of resistance (Cheng & Han, 2020; Elias et al., 2020)

It has been demonstrated that S. aureus-induced mastitis is more common in late breastfeeding than in early lactation and is typically subclinical and chronic. It has been shown that S. aureus infections that happen during the first or second lactation have a more significant impact on milk supply than those that happen during the third and later lactations (Cheng & Han, 2020; Cobirka et al., 2020). Staphylococcal transmission: The main reservoirs are infected udders, but these bacteria have also been found in the udder skin, udder ducts, and udder lesions. Bacteria can enter the udder ducts into

uninfected areas through various factors, such as milkers' hands, equipment they use to clean the udders before and after milking, milking heads, and reservoirs (flies) (Petersson-Wolfe et al., 2010).

6.2.***Streptococcus*** spp.

When cultivated on liquid media, *streptococcus* strains often form in chains or pairs, are spherical or oval, and have a diameter of less than 2 μm. They are Gram-positive, immobile, and do not produce endospores. While nearly every species is facultatively anaerobic, some need extra CO2 to flourish. fermentation-based chemo-organotrophic metabolism. Lactic acid is the primary product of the fermentation of carbohydrates; no gas is produced. negative for catalase. The nutrition needs are varied and complex. L-lysine is the diamino acid at position 3 of the peptide component of peptidoglycan, which is a member of group A. There are no menaquinones. The Lancefield serological grouping system is based on cell wall polysaccharides. The presence of substantial levels of ribitol and the notable absence of rhamnose, a ubiquitous component of nearly all streptococcal cell walls, among members of the Mitis species group, which includes Streptococcus pneumoniae, are essential chemotaxonomic markers for these taxa (Whiley & Hardie, 2015).

The surroundings of dairy cows and the gastrointestinal tract of cows are home to *S. agalactiae*. A recent study has demonstrated that udder protection and milking cleanliness help prevent *S. agalactiae* infection, but fecal and environmental management should also be considered. It can be spread by the milking machine and fecal pathway, primarily through contaminated drinking water. Although no abnormalities exist in the milk, *S. agalactiae* produces subclinical mastitis with high SCC and reduced milk supply. It can live eternally in the mammary glands by creating a biofilm that enables it to stick to the mammary gland and endure while boosting resistance to host factors and food deprivation. Recurrent mastitis linked to both clinical and subclinical infections is caused by the environmental bacterium *S. uberis*. Developing biofilms by the α-casein and β-casein components in milk enables *S. uberis* to withstand antibiotic treatment and environmental stress. Lips, tonsils, skin, oral cavity, rumen, respiratory tract, rectum, teat mouth, infected udders, feces, and wounds are among the animal sections where it has been found (Cheng & Han, 2020).

6.3.***E. coli***

Within the family *Enterobacteriaceae* and tribe *Escherichia*, the genus *Escherichia* comprises predominantly motile Gram-negative bacilli, including *E. coli*. Clinical specimens on general or selective media at 37°C under aerobic conditions can easily yield *E. coli*. MacConkey or eosin methylene blue agar, which selectively cultivates *Enterobacteriaceae* members and enables morphological distinction of enteric organisms, is the primary medium on

which E. coli in feces is recovered (Nataro & Kaper, 1998).

Through the teat, it enters the dairy cow's udder, grows, and triggers an inflammatory reaction. It is present in the dairy cow's immediate surroundings, including the herd bedding, mainly when the weather is damp. *E. coli*-induced mastitis is typically clinical and temporary. The symptoms can be severe with systemic indicators (fever) or mild with only local signs (red and swollen udder). E. coli-induced severe clinical mastitis can result in total loss of milk output, irreparable tissue damage in the mammary gland, and occasionally even a dairy cow's death (Cheng & Han, 2020).

6.4. *Mycoplasma*

It represents a pathogenic species within the genus *Mycoplasma*, belonging to the *Mycoplasmatales* group of the family *Mycoplasmataceae* and the class *Mollicutes*. It stains well with Giemsa but is weakly Gram-negative. It is coccoid-shaped, approximately 0.25-0.5 microns in size. *Mycoplasmas* are small, pleomorphic microorganisms without a cell wall and require special media for their cultivation (Kılıç, 2010).

Compared to *S. aureus* and *S. agalactiae* infections, *Mycoplasma* spp.-induced infectious mastitis is less frequent. It is severe, though, and destroys the glandular tissues, causing the gland and lymph nodes to fibrose and develop abscesses. Mycoplasmal mastitis outbreaks occur occasionally and are not intentionally treated. Despite self-limiting, it creates a biofilm that infiltrates the host cell and is resistant to antibiotics. Rapid separation or culling and routine monitoring of the afflicted are the only controls (Cheng & Han, 2020).

6.5. *Corynebacterium*

Four species of nonlipophilic corynebacteria were identified as being linked to clinical and subclinical mastitis in dairy cows: *C. ulcerans*, *C. pseudotuberculosis*, *C. amycolatum*, and *C. minutissimum*. Mastitis *C. pseudotuberculosis* strains had different colony morphologies and less inhibitory action on staphylococcal β-hemolysin than biovar equivalent, ovis reference strains, and goat field strains. The most commonly isolated non-lipophilic *corynebacteria* was *C. amycolatum*. (Hommez et al., 1999).

There may be handle-shaped forms, ellipsoidal, oval, or, in rare cases, flagellated or thinner rods. The snapping section results in cell patterns that are palisade and angular. Some cells stain erratically, although Gram staining is positive. Certain species may have metachromatic granules. It is not acid-fast. not producing spores. Every species is immobile. Every species possesses catalase. All are oxidase-negative, except *C. aurimucosum*, *C. doosanense*, *C. bovis*, and *C. maris*. While some species are aerobic, many are facultatively anaerobic. There are lipophilic species. Several species use glucose and a few

other sugars in a peptone medium to make acid. While most species do not alkalinize citrate as their only carbon source, few do (Bernard & Funke, 2015).

6.6. *Trueperella pyogenes*

The epidermis and mucosa of animal's urogenital, gastrointestinal, and upper respiratory tracts are frequently home to *T. pyogenes*. This Gram-positive, opportunistic bacterium causes various suppurative diseases in many animal species, including abscesses, arthritis, endocarditis, mastitis, metritis, osteomyelitis, pneumonia, and vasculitis (Peek et al., 2018).

T. pyogenes is one of the most clinically necessary bacteria responsible for severe mastitis and metritis, especially in postpartum dairy cows (Rezanejad et al.,2019; Peek et al., 2018). The bacterium has antibiotic resistance and virulence characteristics. The most prevalent clinical signs in animals are lymphadenitis (9%) and pneumonia (11%), mastitis (45%), and abscesses (18%) (Rogovskyy et al., 2018).

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CHAPTER 5

CRYOPRESERVATION OF SPERM CELLS

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1. Introduction

Semen extenders were created to safeguard sperm from detrimental factors such as freezing, oxidative stress, osmotic shock and damage caused by ice crystal formation. These extenders help maintain sperm quality by stabilizing key characteristics, including sperm morphology, motility, viability, and the integrity of the membrane, DNA and acrosome. To enhance semen quality for fertilization, semen extenders must ensure an optimal pH, provide adenosine triphosphate, protect against cooling and freezing shocks, and possess antioxidant properties (Bustani and Baiee. 2021).

Cryopreservation negatively affects the functionality of sperm cells, potentially leading to diminished fertility. However, this issue can be mitigated by employing various extenders designed to interact with semen and safeguard different cellular components throughout the stages of cooling, freezing, and thawing (Celeghini et all. 2008).

2. Preservation of Semen

Cryopreservation and chilling are the main methods for sperm storage (Bustani and Baiee. 2021). The chilling method provides a simpler and more cost-effective alternative for artificial insemination (AI) due to its ease of storage, dilution, transport, and the efficient restoration of sperm function under field conditions (Raza et all. 2024). On the other hand cryopreservation offers preservation for living cells for extended period of time (Uçan et all. 2016), given that the temperature remains at –196°C in liquid nitrogen at all times (Bustani and Baiee. 2021).

The quality of stored semen is affected by processes like dilution, centrifugation, extender use, and freezing, which can increase the amount of reactive oxygen species (ROS). Since sperm membranes are rich in polyunsaturated fatty acids, they are highly susceptible to lipid peroxidation caused by ROS, resulting in altered membrane fluidity (Filho et all. 2009) and leading to cellular damage that impairs sperm function (Sieme et all. 2016).

The main challenge during cryopreservation is not low temperatures, but the lethal intermediate temperature zone (-15 to -60°C) that cells must pass through twice: during cooling and warming. If cooled too rapidly, cells supercool and freeze intracellularly, which typically kills them. If cooled too slowly, cells dehydrate but may experience damage due to prolonged exposure to high solute concentrations. Both too fast and too slow cooling rates can kill cells, but the mechanisms of damage differ (Gao et all. 1997). The sperm cell nucleus, wich regarded as a stable, less impacted structure by freezing and thawing compared to membranes, can still suffer negative effects from cryopreservation. Even if sperm cells survive the negative effects of cryopreservation and stay motile after being thawed, the damage they took

might make it imposible for them to penetrate the zona pellucida and fertilize an oocyte (Celeghini et all. 2008).

3. Semen Extenders and Their Compounds

An ideal semen extender should be isotonic, containing essential minerals to support sperm viability. It must include cryoprotective agents such as glycerol, DMSO, and ethylene glycol, along with buffers like Tris to eliminate metabolic residues (Aksu. 2023). The extender should preserve the integrity of both the plasma and acrosome membranes and include antibiotics (e.g., penicillin, streptomycin) to prevent bacterial contamination. Common extenders for freezing sperm typically use additional components like sugars and solutes for pH balance (Layek et all. 2016).

Until now, a variety of extenders have been utilized to improve cryosurvival rates while minimizing membrane damage (Uçan et all. 2016). While cryoprotective agents help reduce cryopreservation-induced injury, they are also toxic to cells at high concentrations and should therefore be used with caution (Sieme et all. 2016).

Cryoprotectant compounds can be broadly divided into groups based on their mechanisms of action. Substances like methanol, glycerol, 1,2-propanediol, ethylene glycol, butanediol and DMSO are part of a category that penetrates the cellular cytoplasm (Holt. 2000). Their protective effect primarily stems from their ability to reduce electrolyte concentration during freezing, thereby reducing osmotic shrinkage at a specific temperature. The second category of cryoprotectant compounds consists of non-permeating solutes, including sugars and larger molecules like hydroxyethyl starch (HES), polyvinylpyrrolidone (PVP), polyethylene glycols (PEG), and dextrans. These solutes generally cannot provide protection alone but often enhance the effectiveness of a permeating compounds or allow the use of a lower concentration of it (Gao et all. 1997).

Various extenders utilize different materials, including animal-derived sources like egg yolk and skimmed milk, as well as plant-based sources such as soybean lecithin. These materials offer distinct advantages and pose specific challenges depending on the type of sperm extender and species involved (Bustani and Baiee. 2021).

3.1. Egg Yolk

Egg yolk-based extenders are commonly used for both chilled and frozen semen. Their protective effect against cold shock is attributed to the presence of low concentrations of substances like lecithin and β-lipovitellin, which play a key role during cooling and freezing (Aksu. 2023). Metal chelators like yolk phosvitin, ceruloplasmin, ovalbumin, and ovotransferrin also help remove free metal ions that could otherwise catalyze ROS production (Filho et all. 2009).

Even though egg yolk based semen extenders are widely used in both laboratory and field applications due to their affordability and effective results, these extenders also carries the risk of contamination. Egg yolk can also interfere with sperm assessment, and the presence of particulate matter in the extender may negatively affect fertility (Layek et all. 2016). There is also an issue with standardization, as biological products like egg yolk contain a variety of substances. As a result, they cannot be fully standardized and may vary between batches (Pagl et all. 2006).

3.2. Milk

Milk-based extenders are known to be as effective as egg yolk-based extenders in protecting sperm cells. Skimmed or whole milk can be used as a buffer for sperm preservation, where semen is diluted and can be stored at 4°C or frozen with glycerol (Manjunath. 2012). Lactose, a key compound in milk, is hydrophilic and unable to pass through the sperm cell wall, thereby protecting the cell membrane and preventing freeze shock (Bustani and Baiee. 2021).

Milk-based extenders are recognized for their beneficial impact on enhancing antioxidative activity in extended semen (Pagl et all. 2006). Unfortunately, like egg-based extenders, milk-based extenders also have the problem of particulate matter in the extender (Layek et all. 2016) and the same risk with standardization (Pagl et all. 2006).

3.3. Soybean Lecithin

The potential risk of disease transmission associated with traditional animal-based extenders, such as those containing egg yolk or milk by-products, has raised concerns, leading to a growing focus on the development of semen extenders that do not rely on animal sources (Layek et all. 2016). To answer these concerns, soybean lecithin can be used as an alternative to egg yolk (Aksu. 2023). In several experiments with bulls and boars, soy-based extenders has shown satisfactory results and reduced bacterial contamination in frozen-thawed semen (Bustani and Baiee. 2021).

3.4. Sugars

Sugars are commonly used in sperm extenders as they serve multiple roles, such as supplying an energy source for sperm cells during incubation and maintaining the osmotic pressure of the diluents (Yıldız et all. 2000). To improve post-thaw performance, extenders can be supplemented with different monosaccharides (e.g. glucose, fructose) and/or disaccharides (e.g. sucrose, trehalose). Disaccharides offer an advantage over monosaccharides due to their superior ability to induce osmotic dehydration and prevent intracellular ice crystallization (Uçan et all. 2016).

Among disaccharides, trehalose, which is a non-permeable sugar, plays a crucial role in protecting the plasma membrane from the harmful effects of cell dehydration (Ahmad and Aksoy. 2012). It is important to note, however, that the cryoprotective effectiveness of sugars can vary based on factors such as the type of buffer used, storage temperature and the molecular weight of the sugar (Yıldız et all. 2000).

3.5. Antibiotics

The bacterial load in semen can rise significantly, particularly when the prepuce is not adequately cleaned prior to semen collection or when proper hygiene protocols for equipment are not maintained (Aksu. 2023). A variety of antibiotics can be added in semen extenders, including streptomycin and penicillin, apramycin, ceftiofur, aminoglycosides, or combinations such as linco-spectin with tylosin and gentamicin (Bustani and Baiee. 2021).

3.6. Antifreeze Proteins

Antifreeze proteins (AFPs) are naturally found in various organisms that endure subzero temperatures, like cold-water ocean fish, some plants, insects, sea ice diatoms, snow molds, bacteria, snowalgae and so on. Their ability to inhibit ice recrystallization, combined with their interaction with biological membranes, makes them promising molecules for use in cryopreservation protocols (Feeney and Yeh. 1998)(Robles et all. 2019)(Xiang et all. 2020).

The first study on AFP's in livestock was carried out on sheep sperm, demonstrating that adding 10 μg/mL of AFP to the freezing medium improved both sperm viability and acrosome integrity (Yánez-Ortiz et all. 2022). Various other studies that have followed demonstrated that incorporating AFPs during cryopreservation enhances the viability of sperm cells without impairing their differentiation capacity or functionality. One of the key limitation of AFPs however, is their inability to penetrate cell membranes and protect the intracellular compartment (Tas et all. 2021). Another limitation is due to their difficulty and high cost of isolation, these antifreeze proteins are not currently commercially produced from marine species. Consequently, their application in sperm cryopreservation protocols for farm animals is minimal (Yánez-Ortiz et all. 2022).

AFPs protect against ice through several mechanisms. These include: (a) In freeze avoidance, AFPs lower the freezing point of bodily fluids, preventing the growth of ice crystals through thermal hysteresis, a mechanism observed in fish and insects. (b) In freeze tolerance, AFPs bind to ice surfaces, inhibiting the growth of small crystals, as seen in plants, centipedes, and nematodes. (c) In ice adhesion, AFPs from microorganisms such as *M. primoryensis* facilitate bacterial attachment to ice. These AFPs generally consist of three functional regions: the C-terminal adhesion region, the intermediate repeat region, and the N-terminal cell-anchor region. (Crevel et all. 2002)(Xiang et all. 2020).

Four unique macromolecular antifreezes have been identified and characterized in various marine fish species (Lee et all. 2011). Type I is an alanine-rich, amphiphilic α-helix; Type II is a larger protein, featuring a high content of reverse turns and five disulfide bridges; and Type III is intermediate in size, with no notable features in terms of secondary structure or amino acid composition (Davıes and Hew. 1990). Unlike animal AFPs, plant AFPs have not been fully classified due to immense diversity they have (Bredow and Walker, 2017).

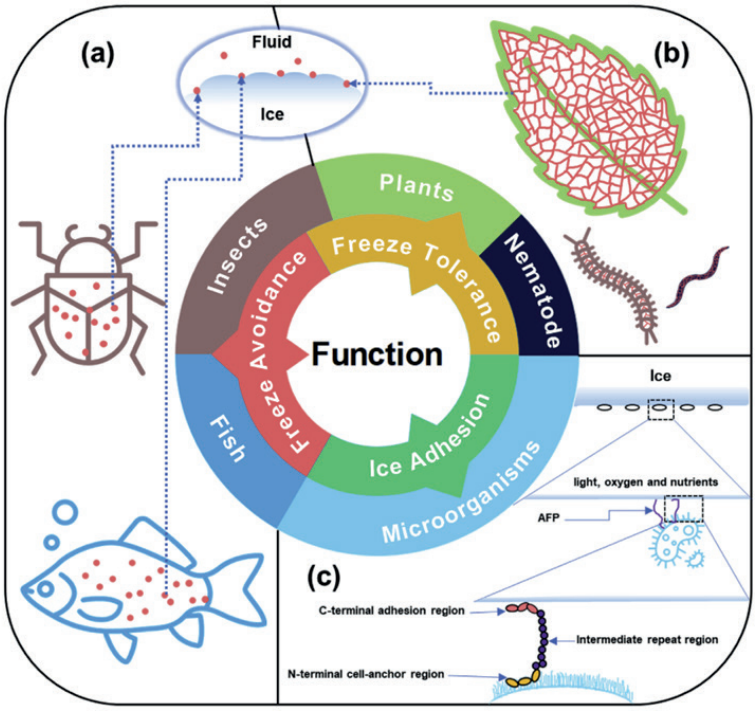

Figure 1. *Biological functions of AFPs in various organisms. (Xiang et all. 2020)*

3.7. Other Added Components

Several plant extracts have been demonstrated to influence fertility, functioning as antioxidants through their ability to scavenge free radicals. Examples include strawberry, green tea, virgin coconut oil, pomegranate, and *Pinus brutia* (Bustani and Baiee. 2021). Other components like honey, fish oil and nano selenium particles also seems to improve post-thawing quality (Aksu. 2023).

Because of their non-enzymatic antioxidant nature, vitamins are another ingredient that can be added to improve semen function parameters of cryopreserved sperm cells (Bustani and Baiee. 2021). Vitamin C has been

shown to enhance the proportion of live, acrosome-intact sperm while reducing the percentage of abnormal sperm cells. Likewise, vitamin E, as a lipophilic molecule integrated into the cell membrane, acts as a membrane stabilizer and a potent antioxidant. It plays a critical role in shielding the cell membrane from lipid peroxidation and damage caused by ROS attacks (Shahin et all. 2020).

Seminal plasma (SP), which is a secretory product from the epididymides, testes and accessory glands, serves as the medium for transporting sperm to the female reproductive tract. It can be corporated to sperm extenders but it has been characterized as both beneficial and detrimental to sperm in different ways (Manjunath. 2012). Sperm cryopreservation protocols usually involve removing SP and replacing it with freezing media. While SP is considered harmful to in vitro sperm survival, its removal reduces sperm motility, metabolic activity, and fertilizing capacity. Thus, adding SP or its components, especially proteins and antioxidants, may also improve sperm cryotolerance (Yánez-Ortiz et all. 2022).

4. Cryopreservation of Semen In Various Species

Spermatozoa vary in size and shape depending on the species, possibly as an adaptation to sperm competition or environmental factors (Prieto et all. 2014). The reaction of spermatozoa to cryopreservation differs both between individual males of the same species and across various species (Küçük et all. 2014). The ratio of cholesterol to phospholipids is viewed as a crucial determinant of sperm plasma membrane fluidity and stability. Species with a lower cholesterol-to-phospholipid ratio in their sperm plasma membranes are typically expected to be more sensitive to cold shock (Ahmad et all. 2013).

For example, the spermatozoa of the boar, bull, and ram are known to be especially sensitive to cold shock. Even though some sources claim that stallion spermatozoa (Weitze and Petzoldt. 1992) and rabit spermatozoa (Ahmad et all. 2021) seem to be less prone to cold shock, others suggests that stallion spermatozoa is not exceptionaly resistant to it (Bustani and Baiee. 2021). It is also known that in cattle, cryopreservation results in a gradual decrease of up to 50% in sperm motility and viability after thawing. In pigs however, while the reduction in motility is similar to cattle, the decline in sperm viability is more significant (~60%) (Yánez-Ortiz et all. 2022).

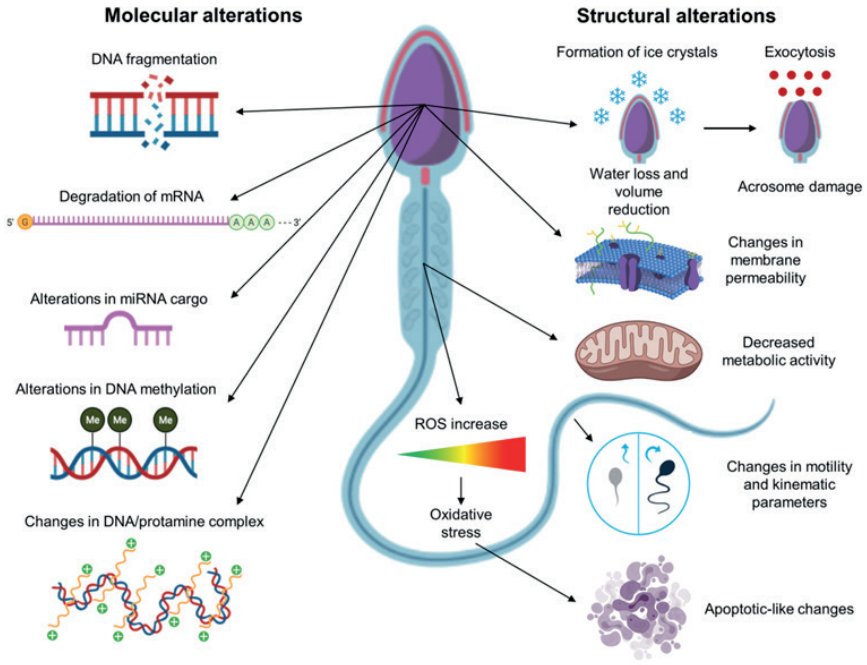

Figure 2. *Changes in mammalian sperm induced by cryopreservation. (Yánez-Ortiz et all. 2022)*

5. Conclusion

The cryopreservation of sperm cells is a multifaceted procedure involving numerous critical stages and factors that significantly influence post-thaw outcomes. The reduced conception rates associated with frozen semen compared to fresh semen pose a considerable challenge to achieving optimal pregnancy rates, highlighting the need for effective strategies to mitigate cryo-injuries.

Semen freezing extenders play a crucial role in protecting sperm from the adverse effects of cryopreservation, such as cold shock, osmotic stress, plasma membrane phase transitions, and water flux. However, each constituent of an extender exerts a distinct effect on sperm parameters, underscoring the complexity of their formulation.

In summary, ongoing research aims to develop advanced semen diluents capable of minimizing or preventing damage during both short-term and long-term semen storage.

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